

# Genomic Insights into Bifidobacteria

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## INTRODUCTION

### Taxonomy of Bifidobacteria

The phylum *Actinobacteria* is one of the largest phyla in the domain *Bacteria*, with 5 subclasses, 6 orders, and 14 suborders, all of which are high-G+C Gram-positive bacteria, except for *Tropheryma whippelii*, which has a 46.3% G+C content (82, 325). In this phylum, the order *Bifidobacteriales* contains two families, including *Bifidobacteriaceae* and *Incertae* (93). The most recent *Taxonomic Outline of Bacteria and Archaea*, release 7.7 (TOBA 7.7), suggests that the *Bifidobacteriaceae* family should be divided into five genera: *Bifidobacterium*, *Aeriscardovia*, *Gardnerella*, *Parascardovia*, and *Scardovia* (93). The addition of the genera *Aeriscardovia*, *Parascardovia*, and *Scardovia* was suggested based on their different DNA G+C contents and partial heat shock protein 60 (Hsp60) gene sequences of three *Bifidobacterium* species, namely, *Bifidobacterium aerophilum*, *B. denticolens*, and *B. inopinatum*, respectively (132, 291). These three species are therefore no longer considered part of the *Bifidobacterium* genus. The addition of the genus *Gardnerella* was proposed based on 16S rRNA gene sequence analysis (298). While 16S rRNA gene sequence analysis is the most common approach for calculating microbial phylogeny, a multigene approach can give a more accurate assessment of phylogeny within a genus. This has proven useful for the *Bifidobacterium* genus, and several genes have been utilized for this purpose, such as *recA* (154), *ldh* (259), and *tuf* (324).

Bifidobacteria were first isolated from the feces of breast-fed infants in 1899, by Henri Tissier, and were designated *Bacillus bifidus* (314, 315). Even though Orla-Jensen proposed the genus *Bifidobacterium* in 1924 (213), bifidobacteria were classified into other taxonomic groups, such as *Bacillus bifidus* (1900), *Bacteroides bifidus* (1923 to 1934, in the 1st to 4th editions of *Bergey's Manual of Systematic Bacteriology* [Bergey's Manual]), and *Lactobacillus bifidus* (1939 to 1957, in the 5th to 7th editions of *Bergey's Manual*), for several decades. In 1973, Poupard et al. (234), and subsequently the 8th edition of *Bergey's Manual* (24), reclassified them as a separate taxon and designated the genus *Bifidobacterium*, consisting of 11 species. Scardovi (269) updated this to 24 species in 1986, and currently there are 31 proposed species that have been isolated from the intestines of humans, animals, and insects, and also from human dental caries and raw milk (Table 1).

Relying solely on a single genetic marker, such as the 16S rRNA gene, for bacterial classification does have limitations. Whole-genome approaches can provide much-more-meaningful taxonomic data. Such phylogenetic analyses have been evaluated for many bacterial genomes and have provided evidence for both organism and gene taxonomic families (36, 41). A phylogenomic analysis of the *Lactobacillus* genus has suggested that GroEL is a better phylogenetic marker for this genus than the 16S rRNA gene (49). Since genome sequences for the *Bifidobacterium* genus are becoming available, a whole-genome phylogenomic approach will provide insights into the classification of this fast-growing genus.

### General Characteristics

Bifidobacteria are nonmotile, non-spore-forming, non-gas-producing, Gram-positive, anaerobic, catalase-negative bacte-

ria with a high G+C content (55 to 67%) (146, 269, 336). Their morphology is generally referred to as bifid or irregular V- or Y-shaped rods resembling branches. The actual reason for the irregular shape of bifidobacteria is not yet clearly understood. However, a few studies have revealed that the absence or low concentrations of *N*-acetylamine-sugar (100),  $\text{Ca}^{2+}$  ions (148–150), or amino acids (alanine, aspartic acid, glutamic acid, and serine) (125) in growth media exclusively induce the bifid shape of bifidobacteria.

### Early Studies of Bifidobacteria

After the discovery of bifidobacteria in the feces of breast-fed infants, Tissier (315) suggested that the large number of bifidobacteria in the feces of healthy breast-fed infants was likely the reason for their lower incidence of infantile diarrhea. In his pediatric work, he used bifidobacteria for the treatment of this intestinal diarrhea, and this likely represents the first example of the oral administration of a live microorganism for the treatment of a disease (316). The abundance of bifidobacteria in the feces of breast-fed infants was thought to be due to the *Bifidobacterium*-stimulating properties of human breast milk (38, 46, 60, 96, 196, 321). Numerous studies have substantiated the higher bifidobacterial counts and lower incidences of gastroenteritis in breast-fed infants than in formula-fed infants (3, 40, 51, 98). Early attempts to make infant formula resemble human breast milk in order to promote bifidobacteria in the intestine were unsuccessful (1, 96). Subsequent studies found the presence of bifidobacterial growth-promoting factors in human breast milk, such as lactulose (151, 231) and *N*-acetylglucosamine-containing saccharides and other human milk oligosaccharides (HMOs) (108, 109), supporting the association between large bifidobacterial numbers and human breast milk. These HMOs consist of short-chain trisaccharides, such as sialyllactose or fucosyllactose, and complex, high-molecular-weight glycans, such as *N*-acetylglucosamine polymers (31). The stimulation of bifidobacteria by human breast milk, resulting in their prevalence in the gut, was proposed to be involved in the suppression of undesirable intestinal bacteria (38). This hypothesis was tested by comparing the compositions of intestinal bacteria in the feces of breast-fed and formula-fed infants, and the proposed increase in bifidobacteria and concomitant decrease in undesirable microbes were observed (352). This inhibition effect by bifidobacteria was supported by fecal pH measurements, as the fecal pH of bottle-fed infants was found to be >7.0, and that of breast-fed infants was <6.0, during the first 7 weeks after birth (39). This is most likely due to the production of lactic and acetic acids by bifidobacteria.

Due to the potential health benefits of bifidobacteria, they have been recommended as dietary supplements by many authors throughout the last century (180, 227, 244, 263, 316). Freeze-dried bifidobacterial preparations, sometimes with *Lactobacillus acidophilus*, have been used for the treatment of gastrointestinal (GI) disorders (235, 246). The addition of lactulose with bifidobacteria in milk was reported to have beneficial effects in the treatment of hepatic encephalopathy (186, 199). Currently, bifidobacteria are added to numerous foods, specifically for their perceived probiotic activities.

TABLE 1. Currently assigned species of the genus *Bifidobacterium*

Species no. <sup>a</sup>	Name <sup>b</sup>	Subspecies	Origin <sup>c</sup>	%G+C <sup>d</sup>	Reference
1	<i>B. adolescentis</i>		Intestine of adult	59.6 ± 0.8	248
2	<i>B. angulatum</i>		Human feces	59.0 ± 0.1	270
3	<i>B. animalis</i>	<i>B. animalis</i> subsp. <i>animalis</i> <i>B. animalis</i> subsp. <i>lactis</i>	Animal feces Yogurt	60.1 ± 0.3 61.9	189 185
4	<i>B. asteroides</i>		Intestine of honeybee	59.0	272
5	<i>B. bifidum</i>		Infant feces	62.3	315
6	<i>B. bombi</i>		Intestine of bumblebee	47.2	140
7	<i>B. boum</i>		Rumen of cattle	60 ± 0.2	273
8	<i>B. breve</i>		Intestine of infant	58.8 ± 0.4	248
9	<i>B. catenulatum</i>		Intestine of adult	54.0 ± 0.2	270
10	<i>B. choerinum</i>		Porcine feces	66.3 ± 0.2	273
11	<i>B. coryneforme</i>		Intestine of honeybee	ND	28
12	<i>B. crudilactis</i>		Raw milk	56.4	68
13	<i>B. cuniculi</i>		Feces of rabbit	64.1 ± 0.4	273
14	<i>B. dentium</i>		Human dental caries	61.2 ± 0.4	270
15	<i>B. gallicum</i>		Human feces	ND	159
16	<i>B. gallinarum</i>		Chicken cecum	65.7 ± 1.5	343
17	<i>B. indicum</i>		Intestine of honeybee	60.0	272
18	<i>B. longum</i>	<i>B. longum</i> subsp. <i>infantis</i> <i>B. longum</i> subsp. <i>longum</i> <i>B. longum</i> subsp. <i>suis</i>	Intestine of infant Intestine of adult Porcine feces	60.5 ± 0.3 60.8 ± 0.8 62.0	248 248 179
19	<i>B. magnum</i>		Rabbit feces	60.0 ± 0.6	274
20	<i>B. merycicum</i>		Bovine rumen	ND	26
21	<i>B. minimum</i>		Sewage	61.5	28
22	<i>B. pseudocatenulatum</i>		Infant feces	57.5 ± 0.3	273
23	<i>B. pseudolongum</i>	<i>B. pseudolongum</i> subsp. <i>globosum</i> <i>B. pseudolongum</i> subsp. <i>pseudolongum</i>	Bovine rumen Porcine feces	63.8 ± 0.4 59.5 ± 0.4	28 346
24	<i>B. pyschraerophilum</i>		Porcine feces	59.2	291
25	<i>B. pullorum</i>		Chicken feces	67.5 ± 0.4	318
26	<i>B. ruminantium</i>		Bovine rumen	ND	26
27	<i>B. saeculare</i>		Rabbit feces	ND	27
28	<i>B. scardovii</i>		Human blood	ND	123
29	<i>B. subtile</i>		Sewage	61.5	28
30	<i>B. thermophilum</i>		Porcine feces	60.0	189
31	<i>B. thermacidophilum</i>	<i>B. thermacidophilum</i> subsp. <i>porcinum</i> <i>B. thermacidophilum</i> subsp. <i>thermacidophilum</i>	Porcine feces Sewage	ND ND	354 77

<sup>a</sup> There are 31 *Bifidobacterium* species to date.

<sup>b</sup> *B. aerophilum*, *B. denticolens*, and *B. inopinatum* were reclassified as *Aeriscardovia aeriphila*, *Parascardovia denticolens*, and *Scardovia inopinata*, respectively.

<sup>c</sup> Environment from which the species was initially isolated.

<sup>d</sup> Mean ± SD. ND, not determined.

### Microbial Diversity and the Role of Bifidobacteria in the Large Intestine

The human large intestine is a very complex ecosystem that is still not fully understood, and while its microbial composition consists primarily of just four bacterial phyla, *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, and *Proteobacteria* (as well as some *Archaea*, *Eukarya*, and viruses), it is highly variable at the genus level between individuals, likely due to factors such as age, health, diet, etc. (25, 99, 127, 244). Therefore, the adaptation capabilities of the intestinal bacteria corresponding to these variable factors likely influence the overall composition of intestinal microflora in the intestine (89).

**Culturing and nonculturing analysis.** The composition of the microflora in the human large intestine, as estimated using culturing techniques, is usually dominated by the genera *Bacteroides*, *Eubacterium*, and *Bifidobacterium*, with several other predominant genera, such as *Clostridium*, *Peptostreptococcus*, *Enterococcus*, *Lactobacillus*, and members of the family *Enterobacteriaceae* (117, 118). The number of species estimated by

culturing techniques is approximately 400. Nonculturing analysis of the gut microflora was greatly facilitated by the direct isolation of DNA from feces and by amplification of the 16S rRNA genes representing the entire microflora. Cloning and sequencing of individual rRNA genes enabled the numerically dominant genera of bacteria to be identified. This molecular analysis of the intestinal microflora in fecal and colonic samples initially suggested that the human large intestine contains more than 500 different bacterial species and that about 75% of them are nonculturable (81, 83). However, a recent extensive metagenomic analysis revealed that this is an overestimation, with most individuals harboring approximately 160 different bacterial species, the majority of which are "known" (239). While the total number of species present in the human gut is not known, recent molecular studies indicate that it is in excess of 1,000 (239, 241), with an upper estimate of 1,150 suggested by an extensive metagenomic analysis of 124 individuals (239).

Unlike the results of culturing studies of the microflora composition in the large intestine, molecular studies utilizing

16S rRNA gene sequencing revealed only a relatively small number of high-G+C Gram-positive *Actinobacteria*, including bifidobacteria (83, 99). It can be argued that the universal primers used to amplify the 16S rRNA gene may preferentially select certain groups of bacteria, and there may also be biases in DNA isolation due to differences in cell wall characteristics. While some metagenomic studies have found similar microbial distributions to those in 16S rRNA gene analyses (99), others have shown the *Actinobacteria* to be more prominent (156, 239). These studies substantiate *Bacteroides* as the major genus of the identifiable gut microbiome in adults and weaned infants, followed by the *Firmicutes* family, primarily the genera *Eubacterium*, *Ruminococcus*, and *Clostridium*, and the genus *Bifidobacterium*. In unweaned infants, there was significant individual variation, but *Bifidobacterium* was generally the most dominant identifiable genus, followed by the members of the *Enterobacteriaceae* family (156). The recent metagenomic analysis of 124 individuals provided a more comprehensive picture of the composition of the human intestinal microbiota as well as the minimum metagenome shared among people (239). Based on this metagenomic analysis, the human intestinal microbiota consists of two major phyla, *Bacteroidetes* and *Firmicutes*, and four other prominent phyla, *Actinobacteria*, *Proteobacteria*, *Fusobacteria*, and *Tenericutes*, although significant individual variations are present. Interestingly, this analysis showed that the *Actinobacteria* phylum, including *Bifidobacterium*, was relatively abundant in all participants, which is quite different from previous reports obtained using 16S rRNA gene sequence analysis (83, 99). This study also revealed an extensive list of bacterial functions, particularly metabolite production, that are important for human health but are encoded only in the metagenome (239). These large-scale studies on the intestinal microflora in different individuals are greatly increasing our knowledge of its diversity and function.

**Role of bifidobacteria in the large intestine.** Our understanding of intestinal microbe-host interactions and their symbiosis is growing, but it is still in its infancy. The role of bifidobacteria in the intestinal microbiota is not known, but studies have suggested a likely beneficial role for the host. However, given their significantly higher incidence in the unweaned infant gut than in adults, they may play a more important role in gut microbiota development than in other gut functions. Studies have also revealed bifidobacterial interactions with other gut microbes. For example, cocolonization of gnotobiotic mice with bifidobacteria and *Bacteroides* revealed that the presence of bifidobacteria expanded the diversity of polysaccharides degraded by *Bacteroides*, suggesting a synergistic effect of bifidobacteria with other intestinal microbiota on digestion of polysaccharides which are indigestible by the host (295). Another possible role of bifidobacteria in the large intestine is the production of water-soluble vitamins, such as many of the B group of vitamins, as shown previously (67) and also substantiated by the genome analysis discussed below. An important role of bifidobacteria in the large intestine is believed to be modulation of certain bacterial groups that may be detrimental to the host. Numerous studies support their competitive abilities against other intestinal microbes (14, 120, 251, 252). In addition, *in vitro* studies have shown that bifidobacteria can produce antimicrobial compounds such as organic acids (269), iron-scavenging compounds (214), and bacteriocins (43,

161, 351). An intriguing function of bifidobacteria in the intestine may be in protection against some immune-based disorders, as numerous studies have shown them to stimulate a host innate immune response (13, 116, 209, 295). These inflammatory and immune defense responses are proposed to be triggered by recognition of commensal bifidobacteria via Toll-like receptors (TLRs) of the host innate immune system (242). Furthermore, cell wall constituents and unmethylated CpG DNA motifs of bifidobacteria are also believed to be involved in modulation of innate immune responses (8, 166).

The role of bifidobacteria in controlling the levels of undesirable bacteria, such as clostridia and *Escherichia coli*, in the intestine is inferred from the reverse correlation of bifidobacterial numbers with these bacteria from clinical feeding studies (6, 45, 176). This correlation is also evident in the intestinal microbial changes that occur as people grow older: specifically, the number of bifidobacteria decreases significantly, while the number of other bacteria, including clostridia and *E. coli*, increases (127, 190). This correlation has been substantiated by both culturing and nonculturing methods, using fluorescently labeled probes that detect specific microbes directly in feces (121). The levels of bifidobacteria may also correlate with lower levels of putrefactive products, as the levels of ammonia, indole, *p*-cresol, and phenol, as well as enzymes involved in their production (urease, tryptophanase, and  $\beta$ -glucuronidase), were very low in an infant whose fecal microflora consisted of 96% bifidobacteria compared to those in an adult with a bifidobacterial concentration of 19% (127). This suggests that the balance of the intestinal microflora by supplementation of bifidobacteria is important for maintaining intestinal health (127).

### Potential Health Benefits

Numerous studies have suggested that the presence of bifidobacteria in the human large intestine is associated with many human health benefits. However, some of these studies have been criticized for poor design and weak conclusions. This section critically analyzes the scientific credibility of the involvement of bifidobacteria in these possible health benefits.

**Prevention of diarrhea.** The prevention and treatment of diarrhea were the first proposed benefits of bifidobacteria (316). Some studies have since examined the role of bifidobacterial supplementation in protection from diarrhea onset and from the most prevalent cause of viral diarrhea, rotavirus (Table 2). The majority of these studies have used animal models, and few show convincing statistically significant data for large groups. The human studies that have been conducted have been either not statistically significant or barely so. While bifidobacteria are likely to protect the intestine from diarrheal diseases, the added protective effect of taking bifidobacterial probiotics is not very convincing based on current studies. It is possible that individuals with high levels of bifidobacteria may not benefit from extra supplementation, and since initial bifidobacterial levels were not considered in any of these studies, this may have resulted in the underwhelming effects.

**Establishment of a healthy microflora in premature infants.** The rapid establishment of a healthy intestinal microflora in premature infants is believed to be important for mucosal host



TABLE 2. Summary of clinical studies linking bifidobacteria with potential health benefits

Health benefit	Study summary	Reference
Prevention of diarrhea	Large murine study (29 to 41 mice per group) showing a statistically significant ( $P < 0.001$ ) protective effect of <i>B. bifidum</i> and <i>B. longum</i> subsp. <i>infantis</i> supplementation against rotaviral diarrhea	238
	Large murine study (22 to 46 mice per group) with non-statistically significant data, but proposing a protective effect of heat-killed <i>B. breve</i> against rotaviral diarrhea	347
	Large murine study (52 to 111 mice per group) showing a small, statistically significant ( $P < 0.05$ ) protective effect of <i>B. bifidum</i> supplementation, with reduced shedding of rotavirus ( $P < 0.01$ )	80
	Small piglet study (8 or 9 piglets per group) showing a moderate, statistically significant ( $P < 0.01$ ) protective effect of <i>B. animalis</i> subsp. <i>lactis</i> against all forms of diarrhea and a higher titer of antirotaviral antibodies in the feces	288
	Large human study (26 or 29 infants per group) showing a barely statistically significant ( $P = 0.035$ ) protective effect of <i>B. bifidum</i> and <i>Streptococcus thermophilus</i> supplementation, with reduced shedding of rotavirus ( $P = 0.025$ )	262
	Large human study (44 or 46 infants per group) showing a non-statistically significant protective effect of <i>B. animalis</i> subsp. <i>lactis</i>	47
	Large human study (464 or 449 infants per group) with <i>B. breve</i> and <i>S. thermophilus</i> supplementation; did not show a decrease in number of diarrhea episodes over a 5-month period but did show a reduced severity ( $P < 0.01$ )	312
	Small preterm pig study (5 to 13 pigs per group) showing some reduction ( $P < 0.05$ ) in the incidence of necrotizing enterocolitis from supplementation with <i>B. animalis</i> and 4 species of <i>Lactobacillus</i>	290
Establishment of a healthy microflora in premature infants	Small human study (10 preterm infants per group) showing that supplementation with <i>B. breve</i> resulted in establishment of a bifidobacterial flora in the majority of infants during the first week of life, whereas it took the control group several weeks, with only 3 of 9 infants showing bifidobacteria by week 7	167
	Large human study (33 preterm infants per group) showing that supplementation with <i>B. breve</i> reduced ( $P < 0.05$ ) fecal butyric acid levels, but only in the subgroup of infants that weighed $< 2,500$ g	342
Colon regularity	Small human study (17 subjects with irritable bowel syndrome [IBS] per group) showing some reduction ( $P < 0.05$ ) in colonic transit times from supplementation with <i>B. animalis</i> subsp. <i>lactis</i> and yogurt cultures	2
	Large human study (132 or 135 subjects with IBS per group) showing no statistically significant reduction in colonic transit times, except in a small subset of 19 subjects who had $< 3$ stools/week initially, from supplementation with <i>B. animalis</i> subsp. <i>lactis</i> and yogurt cultures	106
	Small human study (15 or 17 women per group) showing some reduction ( $P < 0.05$ ) in colonic transit times from supplementation with <i>B. animalis</i> subsp. <i>lactis</i>	172
	Large human study (100 elderly subjects), without a control group, showing a reduction ( $P < 0.001$ ) in colonic transit after 2 weeks of supplementation with <i>B. animalis</i> subsp. <i>lactis</i>	184
Lactose intolerance	Small human study (15 people with lactose intolerance) showing some reduction ( $P < 0.05$ ) in breath hydrogen from supplementation with <i>B. longum</i>	133
	Small human study (11 Chinese individuals with lactose intolerance) showing some reduction ( $P < 0.05$ ) in symptom scores from supplementation with <i>B. animalis</i> subsp. <i>lactis</i> and yogurt cultures	114
Cholesterol reduction	Small human study (7 subjects per group) showing some reduction ( $P < 0.05$ ) in serum cholesterol levels following supplementation with <i>B. animalis</i> subsp. <i>lactis</i> and <i>L. acidophilus</i>	15
	Small human study (11 or 18 women per group) showing no reduction in total cholesterol but an increase in high-density lipoprotein (HDL) levels ( $P = 0.001$ )	139
	Medium-sized human study (37 women), without a control group, showing that supplementation with <i>B. longum</i> and <i>L. acidophilus</i> did not affect cholesterol levels	102
Immunostimulatory effects	Small mouse study (5 mice per group) showing some reduction ( $P < 0.05$ ) in CD4 <sup>+</sup> T cells in the spleen and colon following supplementation with <i>B. bifidum</i>	142
	Small mouse study (10 mice per group) showing some reduction in the proinflammatory cytokines IFN- $\gamma$ , TNF- $\alpha$ , and IL-12 from supplementation with <i>B. longum</i> subsp. <i>infantis</i>	182
	Small mouse study (5 mice per group) showing some increase ( $P < 0.05$ ) in mucosal IgA following supplementation with <i>B. longum</i>	303
	Large human study (5 groups of 13 to 15 subjects receiving different amounts of <i>B. animalis</i> subsp. <i>lactis</i> and <i>Lactobacillus paracasei</i> ) showing no statistically significant changes in cytokine levels	48
	Small human study (12 or 13 elderly subjects per group) showing some increase ( $P < 0.05$ ) in the anti-inflammatory cytokine IFN- $\alpha$ and in phagocytic activity following 6 weeks of supplementation with <i>B. animalis</i> subsp. <i>lactis</i>	13

Continued on following page

TABLE 2—Continued

Health benefit	Study summary	Reference
	Small human study (8 subjects with ulcerative colitis per group) showing no significant change in symptom scores but showing some decrease ( $P < 0.05$ ) in expression of genes encoding human proinflammatory cytokines from supplementation with <i>B. longum</i> , inulin, and fructooligosaccharides	90
	Large human study (77 subjects with IBS receiving $1 \times 10^{10}$ live cells of <i>B. longum</i> subsp. <i>infantis</i> ) showing reductions in symptom scores and in the ratio of IL-10 to IL-12 (anti-inflammatory to proinflammatory cytokines), normalized to that of healthy individuals	210
Cancer prevention	Medium-sized mouse study (12 mice per group) showing some reduction ( $P < 0.01$ ) in the incidence of tumors when heat-killed <i>B. infantis</i> cells or cell wall preparations were injected into mice along with tumor cells, with a significant increase ( $P < 0.001$ ) in the number of mice that were cured of tumors	279
	Large mouse study (4 groups of 15 mice) showing some decrease ( $P < 0.05$ ) in carcinogen-induced aberrant crypt foci following supplementation with <i>B. longum</i> and a significant decrease ( $P < 0.001$ ) following cosupplementation with <i>B. longum</i> and inulin	257
	Large mouse study (30 mice per group) showing a significant reduction ( $P < 0.001$ ) in carcinogen-induced colonic neoplasms following supplementation with both <i>B. animalis</i> subsp. <i>lactis</i> and resistant starch but no reduction with either supplement individually	163
	Large human study (4 groups of 18 to 22 colon cancer or polypectomized patients) showing some improvement ( $P < 0.05$ ) in epithelial barrier function and cell toxicity only in polypectomized patients following supplementation with <i>B. animalis</i> subsp. <i>lactis</i> , <i>L. rhamnosus</i> , and inulin	240

defense and the prevention of certain intestinal infections (60). Some studies have been conducted to evaluate the effect of early oral supplementation with bifidobacterial probiotics on preterm infants (Table 2). It appears that early supplementation does decrease the time for a bifidobacterial population to develop, but this would be expected when large numbers are ingested into an immature gut. While this may offer some protective effects, supplementation with *Bifidobacterium longum* and *Lactobacillus rhamnosus* was not found to reduce the time needed for a feeding tube in a study of 45 infants compared to a 49-infant control group (256).

**Colon regularity.** Bifidobacteria may also be useful for the treatment of constipation in the elderly, as their small population of bifidobacteria may be a contributing factor. This was first suggested by the observation of subjects who ingested bifidobacteria and reported a noticeable lubricant effect during fecal passage (216). There have been a number of recent studies which appear to support some effect of bifidobacterial supplementation on reducing colonic transit time in individuals who suffer from long colonic transit times (Table 2). While these studies do suggest a role for bifidobacterial supplementation in colonic transit, they tended to focus on individuals who already had GI problems, not on healthy people. In addition, there was no attempt in any study to understand the mechanism of action. A likely mechanism of action is the production of exopolysaccharides (EPS), which may function as a laxative.

**Lactose intolerance.** Although dairy foods are recommended components of a healthy diet, some people have discomfort digesting dairy products due to a shortage of the enzyme lactase (58, 124). Fermented foods appear to be more suitable for lactose-intolerant patients than nonfermented foods because of the decreased lactose concentration. Some studies have suggested that the addition of large numbers of lactose-digesting cultures, such as bifidobacteria, to dairy products may also alleviate the symptoms of lactose intolerance (Table 2). While these studies are quite small and the results are statistically

significant only at a  $P$  level of  $<0.05$ , this effect is generally accepted as scientifically sound, presumably because the mechanism of action is clear.

**Cholesterol reduction.** Some studies have been conducted to evaluate if supplementation with bifidobacteria can reduce serum cholesterol levels (Table 2). While some of these studies suggest an effect, it is not generally accepted as significant. Part of the reason is the difficulty in obtaining an accurate baseline cholesterol measurement, as levels are known to fluctuate. This calls the statistical significance of effects into question. A possible mechanism has been proposed, as some bifidobacteria have been shown to assimilate cholesterol into their cell membranes (62).

**Immunostimulatory effects.** A number of studies have suggested that the interaction between bifidobacteria and host mucosal cells can result in immunomodulatory effects in the host (Table 2). While the studies are generally small and only just statistically significant, they do suggest some effect, although the one large human study did not show a significant difference. This would be weak evidence, at best, if the effect was mechanistically unclear. However, it is known that the immune system responds to microbes in the gut, and recent molecular evidence obtained using transcription profiling has shown that cecal epithelial cells respond differently to *B. longum*, which specifically increases gamma interferon (IFN- $\gamma$ ), and *Bacteroides thetaiotaomicron*, which induces tumor necrosis factor alpha (TNF- $\alpha$ ) (295). While both of these cytokines are proinflammatory cytokines, these findings do demonstrate that different bacteria have different immunomodulatory stimulatory effects.

**Cancer prevention.** Cancers of the gut are prevalent and are believed to have both genetic and dietary causes. Some studies have examined the potential ability of bifidobacterial supplementation to prevent cancers (Table 2). While these are mainly mouse studies, they suggest that the combination of bifidobacteria and a prebiotic may reduce the probability of carcinogen-

TABLE 3. Various selective media for isolation and enumeration of bifidobacteria

Medium	Selectivity	Reference(s)
<b>Antibiotic-based media</b>		
NPNL	Neomycin, paromomycin, nalidixic acid, LiCl	311
BIM-25	Polymyxin B, nalidixic acid, iodoacetic acid, 2,3,5-triphenyltetrazolium chloride	197
MRS (TPY) + Dic	Dicloxacillin	297
RMS	Neomycin, paromomycin, LiCl, sodium propionate	267
AMC	Polymyxin B, nalidixic acid, iodoacetic acid, 2,3,5-triphenyltetrazolium chloride	11, 12
BL-OG	Oxgall, gentamicin	168
BSM	Mupirocin	164
<b>Antibiotic-free media</b>		
mCAB <sup>a</sup>	Propionic acid	19
LP	LiCl, sodium propionate	158
TP	Transgalactooligosaccharide, sodium propionate	131
RB	LiCl, sodium propionate	112
RAF 5.1	LiCl, sodium propionate	258
BFM	LiCl, propionic acid, methylene blue	204
RCA-aniline blue	Propionic acid, aniline blue	169

<sup>a</sup> Beeren's medium.

induced cancerous cells in mice. However, there are no human trials with convincing evidence showing a protective effect of this type of supplementation.

While numerous studies have found health benefits of ingesting bifidobacteria, some studies have not been able to demonstrate benefits. Differences are believed to be due to the different strains of bifidobacteria used, as many of these effects may be dependent on the strain used as well as on host-specific characteristics. The selection of proper strains and further understanding of the microbial traits necessary for specific health benefits are needed to optimize their potential.

### Detection and Identification

Three principal approaches have been developed for isolation, detection, and identification of bifidobacteria from feces: traditional culturing methods using selective media for selection and identification, culture-free molecular methods for detection, and molecular methods for identification and differentiation.

The first selective medium developed for bifidobacteria was lactose-cystine-liver (LCL) agar (30), but it was inhibitory to some bifidobacterial strains. Norris et al. (207) improved *Bifidobacterium*-selective media by including growth factors (pancreatin, sorbitan monooleate, ascorbic acid, and vitamin B<sub>12</sub>), and these were inhibitory to other intestinal bacteria, except for enterococci. The identification of specific bifidobacterial growth factors from human milk further enhanced the recovery of bifidobacteria (108, 109). In the last few decades, various selective media have been developed and are listed in Table 3. Some are based on the use of antibiotics, such as neomycin, polymyxin B, nalidixic acid, and/or mupirocin, for selectivity, while others utilize inhibitory agents such as lithium chloride and propionate.

The advent of molecular tools has greatly enhanced approaches for detection, differentiation, and identification of bifidobacteria. Many of these are cultivation-free methods allowing *in situ* analysis, thus removing the limitations of culturing. The development and use of these molecular tools for bifidobacteria are summarized in Table 4.

## PLASMIDS OF BIFIDOBACTERIA

### Plasmid Analysis

Plasmids are not commonly detected in bifidobacteria, with approximately 20% of isolated bifidobacteria containing detectable plasmids (281). Currently, plasmids have been detected in 8 of the 31 species of *Bifidobacterium*, including *B. longum* subsp. *longum*, *B. pseudolongum* subsp. *globosum*, *B. indicum*, *B. asteroides* (282, 283), *B. breve* (128), *B. bifidum* (287), *B. catenulatum* (5), and *B. pseudocatenulatum* (97). Some plasmids have been sequenced completely, revealing primarily rolling circular plasmids varying in size from 1.8 to 10.2 kb (Table 5).

Although the majority of the replication (Rep) proteins identified for plasmids from bifidobacteria are homologous to rolling circle replication (RCR) Rep proteins, a few plasmids encoding proteins that are homologous to theta replication proteins have been reported (5, 53, 162). For a comparative analysis of replication proteins encoded by bifidobacterial plasmids, a phylogenetic analysis of 26 Rep proteins from bifidobacterial plasmids and 15 Rep proteins from closely related plasmids was performed, and it categorized bifidobacterial plasmids into five groups based on their Rep proteins (Fig. 1). This grouping is consistent with previous studies on bifidobacterial plasmids (53, 104). The Rep proteins in group I share the conserved Rep<sub>3</sub> domain from the Pfam database (PF01051), and this is the most commonly detected replicon in bifidobacterial plasmids. This domain is also found in replication proteins from many plasmids of the lactic acid bacteria (LAB), and these plasmids replicate via the RCR mechanism (69). Five of the plasmids in this group, pKJ50, pKJ36, pNAC1, pNAC2, and pNAL8L, were shown experimentally to accumulate single-stranded intermediates in bifidobacteria, consistent with the RCR mode of replication (53, 104, 212, 225). Group II Rep proteins, containing the RepA\_C domain (PF04796), are found in a few *B. longum* and *B. asteroides* plasmids. One of these plasmids, pDOJH10L, was shown to replicate via RCR, suggesting that the replication mode of plasmids in group II may follow RCR (162). This was further analyzed

TABLE 4. Molecular detection and identification methods used for bifidobacteria

Method <sup>a</sup>	Description	Reference(s)
DNA fingerprinting methods		
PCR approaches		
AP-PCR	Use of a single arbitrary primer to obtain strain-specific banding patterns; can be subject to reproducibility problems	341, 344
TAP-PCR	Triplicate AP-PCR incorporating three different annealing temperatures to improve reproducibility of profiles	59
ERIC-PCR	AP-PCR using ERIC-PCR primers	289, 333
ARDRA	RFLP analysis of the <i>ldh</i> gene	259
	RFLP analysis of the 16S rRNA gene	153, 330
PFGE	Band profile analysis of complete genome by use of rare-cutting enzymes	292, 338
Molecular identification by sequence analysis of:		
16S rRNA gene		136, 174, 338
<i>groEL/groES</i>		329
<i>recA</i>		154
<i>grpE/dnaK</i>		339
<i>ldh</i>		259
<i>tuf</i>		324
<i>atpD</i>		326
Transaldolase gene		245
Molecular quantification by RT-PCR	Real-time PCR quantification of bifidobacteria in feces, using genus- or species-specific primers	245
FISH	Hybridization of fluorescently labeled genus/species-specific probes to bifidobacteria in feces	157

<sup>a</sup> AP-PCR, arbitrarily primed PCR (also referred to as random amplified polymorphic DNA [RAPD] analysis); ERIC-PCR, enterobacterial repetitive intergenic consensus sequence PCR; ARDRA, amplified rRNA gene restriction analysis; RFLP, restriction fragment length polymorphism; PFGE, pulsed-field gel electrophoresis; FISH, fluorescent *in situ* hybridization.

using a deletion analysis of pCIBAO89 from *B. asteroides*, defining the minimal replicon and demonstrating binding of the Rep protein by gel mobility shift DNA binding assays (55). The Rep protein of p4M from *B. pseudocatenulatum* belongs to group III and contains the Viral\_Rep domain from the Pfam database (PF02407), which is found in some viral replication proteins. All plasmids from *B. breve* and *B. globosum* belong to group IV, whose encoded proteins contain the Rep domain (PF01446) and likely are also involved in RCR, given that single-stranded DNA intermediates were detected for one plasmid from this group (212). Interestingly, the Rep proteins encoded by the group V plasmids pMB1, pDOJH10S, and pBC1, containing the replicase domain (PF03090), are very similar to the theta replicases from *Rhodococcus* (173), *Propionibacterium* (138), *Corynebacterium* (310), and even *E. coli* (305), suggesting that these plasmids may replicate via theta replication (5, 53, 162). This was subsequently substantiated by demonstrating that pDOJH10S does not accumulate single-stranded DNA intermediates during its replication (162). Based on sequence phylogenetic analysis and G+C content, it was proposed that the *B. longum* plasmid pDOJH10S may have originated from *Rhodococcus rhodochrous* (162) (Fig. 1).

Mobilization (Mob) protein genes and origins of transfer (*oriT*), which are involved in plasmid transfer, are frequently found in many bifidobacterial plasmids, suggesting that they may be mobilizable. However, this has not yet been demonstrated. The *oriT* in bifidobacterial plasmids is highly conserved and consists of a DNA sequence (5'-TAAGTGC GCCCT-3') and an inverted repeat, consistent with *oriT* motifs of other plas-

mids (50, 162). Mob proteins have a highly conserved motif (XPHuHuuuXXu, where "u" represents a hydrophobic amino acid) that was previously implicated in the nicking of *oriT* (126).

G+C content analysis of the sequenced plasmids revealed that most have quite different G+C contents from those of their host chromosomes, suggesting that they may have originated from other microorganisms in the recent evolutionary past (Table 5). Since only 40% of the plasmids contain a G+C content within 2% of that of the host chromosome, it was suggested that bifidobacteria can readily accept plasmids from other organisms by horizontal gene transfer. Since the plasmid G+C content ranges from 41.7 to 66.2%, the range of potential plasmid donors must be quite large. Given that all of the plasmids contain *mob* genes but no *tra* genes, it is possible that they were obtained by utilizing the Tra functions of a helper plasmid either in the donor or in another helper organism. All of the sequenced bifidobacterial plasmids to date are cryptic. Only one possible function connected to bifidobacterial plasmids has been reported, as the 8-kb unsequenced plasmid from *B. bifidum* NCFB 1454 was proposed to be involved in bifidocin B production (351). The curing of this plasmid corresponded with the loss of bacteriocin production, but bacteriocin immunity was still retained in those strains. While this suggests the involvement of a bifidobacterial plasmid in bacteriocin production, it has yet to be substantiated. Another recent study linked production of a new bacteriocin, bifidin I, to a plasmid in *B.*



TABLE 5. Completely sequenced plasmids from bifidobacteria

Host species (G+C content [%] <sup>a</sup> )	Plasmid	Size (bp)	G+C content (%)	Host G+C content (%) <sup>b</sup>	Reference or GenBank accession no.
<i>B. longum</i> subsp. <i>longum</i> (60.8 ± 0.8)	pNAC2	3,684	64.7		53
	pTB6	3,624	65.1		309
	pB44	3,624	65.1		287
	pKJ36	3,625	65.1		224
	pMG1	3,682	65.1		222
	pBLO1	3,626	64.8	60.1	275
	P6043B	3,680	65.1		DQ458911
	pNAC1	3,538	58.8		53
	pNAL8L	3,489	59.0		104
	pKJ50	4,960	61.8		225
	pNAL8 M	4,910	61.9		104
	pBIFA24	4,892	61.8		226
	p6043A	4,896	61.8		DQ458910
	pNAC3	10,224	62.0		53
	pDOJH10L	10,073	62.2	60.2	162
	pMB1	1,847	62.0		178
	pDOJH10S	3,661	66.2	60.2	162
	pFI2576	2,197	61.9		194
<i>B. breve</i> (58.8 ± 0.4)	pCIBb1	5,750	56.9		212
	pNBb1	2,297	58.7		E17316
	pB21a	5,206	56.3		287
<i>B. pseudolongum</i> subsp. <i>globosum</i> (63.8 ± 0.4)	pASV479	4,815	59.3		268
<i>B. bifidum</i> (62.3)	pB80	4,898	61.9		287
	pBIF10	9,275	41.7		DQ093580
<i>B. asteroides</i> (59.0)	pCIBAO89	2,111	52.3		55
	pAP1	2,140	52.3		Y11549
<i>B. catenulatum</i> (54.0 ± 0.2)	pBC1	2,540	63.7		5
<i>B. pseudocatenulatum</i> (57.5 ± 0.3)	p4 M	4,488	53.1		97

<sup>a</sup> Host G+C contents were obtained from Table 1, except for those for pBLO1 (from *B. longum* subsp. *longum* NCC2705), pDOJH10L, and pDOJH10S (from *B. longum* subsp. *longum* DJO10A).

<sup>b</sup> G+C contents of completely sequenced host chromosomes.

*longum* subsp. *infantis* BCRC 14602 (43). However, the results of the study do not support the presence of a plasmid in this strain.

### Development of Cloning and Expression Vector Systems for Bifidobacteria

A number of shuttle cloning vectors for bifidobacteria with *E. coli* plasmid replicons have been constructed, and electroporation procedures for bifidobacteria have been developed (4, 162, 177, 178, 188, 222, 225, 254, 255, 268, 287). The high-copy-number ColE1-based replication origin of *E. coli* was used for the construction of all except one of the *E. coli*-*Bifidobacterium* shuttle cloning vectors (4, 177, 178, 225, 255, 287, 309). Interestingly, replication problems in bifidobacteria occurred for two of the vectors, which encoded almost identical Rep proteins (pNAC1 and pNAL8L group I Rep proteins) (Fig. 1), suggesting incompatibility issues with ColE1 (53, 104). Vectors encoding other group I Rep proteins did not exhibit replication issues, suggesting that this incompatibility issue is confined to a small conserved subgroup of group I rep genes.

One vector was constructed using the *E. coli* p15A *ori* instead of ColE1. This has a lower copy number in *E. coli*, which can facilitate cloning of DNA regions that may have stability

issues in *E. coli*. Given that this vector, pDOJHR, also contained a theta replicon from bifidobacteria, it was found to be a stable vector in bifidobacteria, exhibiting high segregational and structural stability without antibiotic pressure (162). Given the low electroporation frequencies for plasmid transfer into bifidobacteria, a number of studies on optimization of electroporation were undertaken, with limited success (9, 253). Recently, methylating plasmids to overcome restriction systems in bifidobacteria were found to greatly improve transformation efficiencies (195, 348).

Several promoter-screening, gene expression, and secretion vectors have been constructed. The reporter gene *gusA*, encoding the *E. coli* β-glucuronidase enzyme, has been used to monitor promoter activity in bifidobacteria (147, 268). A strong 16S rRNA gene promoter from bifidobacteria was used for the expression of a cholesterol oxidase gene from *Streptomyces coelicolor* in *B. longum* (221). The α-amylase gene from *B. adolescentis* INT-57 was cloned into *B. longum*, resulting in extracellular α-amylase production (130, 249). Since the secretion and expression signals from α-amylase were functional, they were used to construct an expression and secretory vector, pBESAF2, which was used to express phytase (223), pediocin (193), and γ-aminobutyric acid (GABA) (220). Currently, no

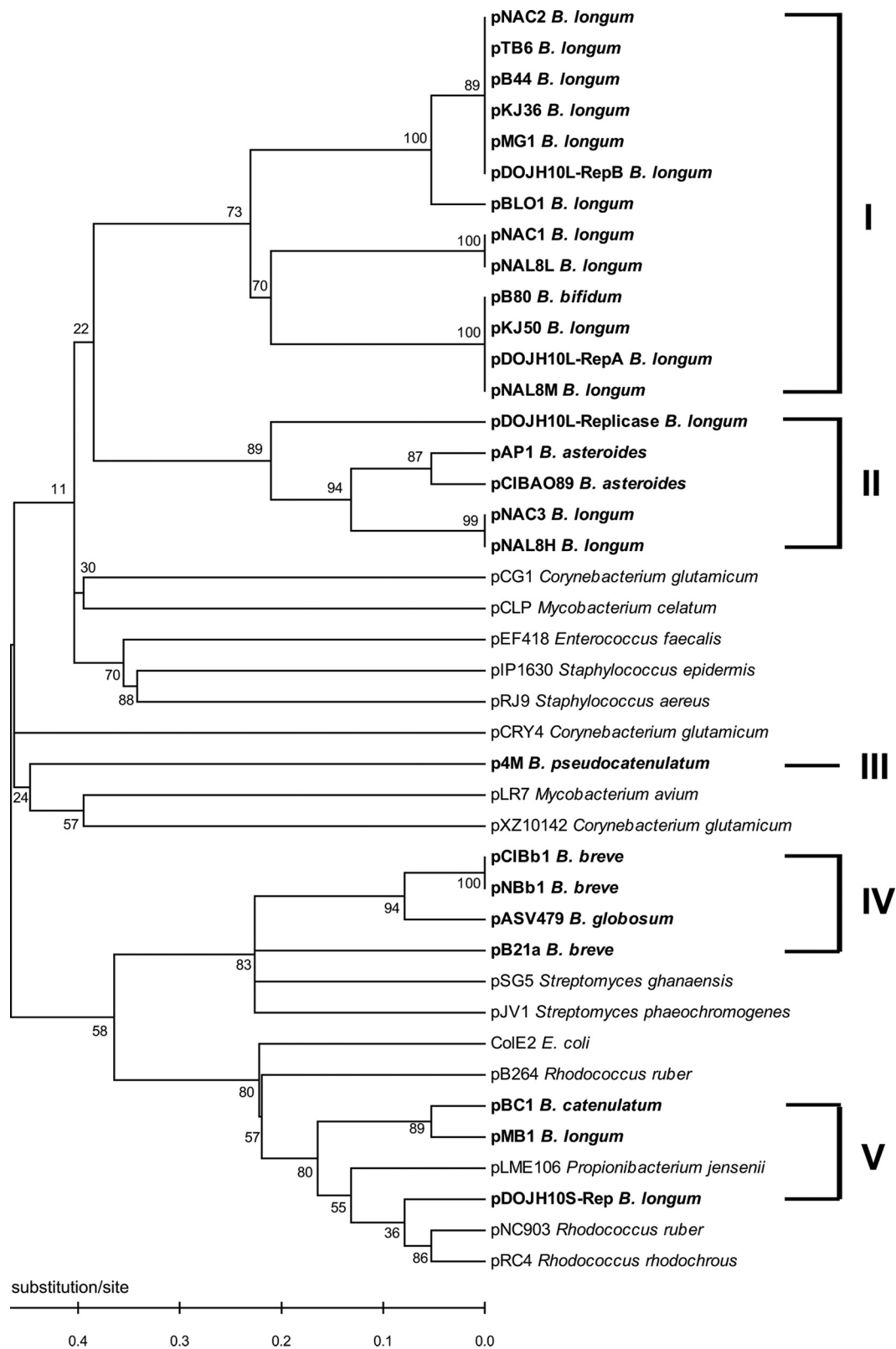


FIG. 1. Phylogenetic analysis of replication proteins expressed by bifidobacterial plasmids. The replication proteins encoded by bifidobacterial plasmids and other homologous replication proteins were compared by ClustalW multiple alignments (313). A phylogenetic tree was generated by the neighbor-joining method, using *P* distance values (307). The numbers associated with the branches represent the bootstrap values. Bifidobacterial plasmid replicons are indicated in bold. Roman numerals indicate the five different classes of replication proteins expressed from bifidobacterial plasmids.

expression vectors with food-grade selection markers have been developed for bifidobacteria.

A vector based on the group V theta-replicating plasmid pBC1 (Fig. 1), containing a *luxABCDE* operon that originated from *Photobacterium luminescens* but was optimized for expression in Gram-positive bacteria (237), was modified for expression of *lux* in *Bifidobacterium breve* (57). This was used for monitoring of bifidobacteria both *in vitro* and *in vivo* in a mouse system. However, cell numbers *in vivo* were not sufficient to allow real-time monitoring of the ingested bifidobacteria. Future optimization of *lux* expression in bifidobacteria may increase the sensitivity of this tool.

Another bifidobacterial expression vector system utilized the *hup* (histone-like protein) gene promoter and was used to express cytosine deaminase in *B. longum* (202), human interleukin-10 (IL-10) in *B. longum* (86), the *Salmonella* flagellin gene in *B. animalis* (304), and human basic fibroblast growth factor (FGF-2) in *B. breve* (286). The expression of cytosine deaminase in *B. longum* was used to treat hypoxic tumors in mice, as previous studies had indicated that bifidobacteria selectively localized only in hypoxic tumor cells, not normal cells, pointing to the tumor-targeting properties of bifidobacteria (56, 165, 349, 350). The function of cytosine deaminase in tumor control is to convert the nontoxic compound 5-fluorocytosine to 5-fluorouracil, which is cytotoxic (202). This vector system was based on a group I replicon from bifidobacteria (pTB6) (Fig. 1) and exhibited stability problems in *B. longum*, but it was more stable in *B. breve* (115). A point mutation in the CD gene, at the active site, was found to increase CD activity 10-fold, thus improving the efficacy of this antitumor activity (111). An analogous antitumor strategy was developed for expression of a human endostatin gene in *B. longum* and *B. adolescentis*, using the lambda P<sub>R</sub>P<sub>L</sub> promoter and a theta-replicating *ori* from the group V bifidobacterial plasmid pMB1 (Fig. 1). This was found to be effective in the treatment of mouse liver tumors (165, 345).

## GENOMES OF BIFIDOBACTERIA

### General Characteristics of Bifidobacterial Genomes

Nine complete genome sequences, for *B. longum* subsp. *longum* (GenBank accession numbers AE014295 and CP000605), *B. adolescentis* (AP009256), *B. longum* subsp. *infantis* (CP001095), *B. dentium* (CP001750), and *B. animalis* subsp. *lactis* (CP001213, CP001515, CP001606, and CP001853), have been obtained to date and are publically available in the GenBank database (17, 92, 141, 161, 275, 280, 335). In addition, 32 genome sequencing projects with other bifidobacteria are currently ongoing (Table 6).

An overall comparison of the nine completed genome sequences revealed genome sizes ranging from 1.9 to 2.8 Mb (Table 7). Interestingly, the smallest genomes belong to the four strains of *B. animalis* subsp. *lactis*. Since strains of this subspecies are believed to have evolved from the *B. animalis* species in a dairy fermentation environment, this substantiates the tendency of bacteria to undergo genome reduction when exposed to less complex environments (206). This is further substantiated by the case of *B. animalis* subsp. *lactis* AD011, which was reported to have only two complete RNA operons, compared

to four or five operons in the other genomes. While this may be an anomaly due to possible sequence assembly errors, it may also be the result of adaptation to a simple, constant-nutrient environment, given that extra rRNA operons allow bacteria to adapt more quickly to new nutrient sources (52, 145).

The nine complete bifidobacterial genomes can be divided into three groups based on phylogenetic analysis of their 16S rRNA genes, including the *B. longum* group (*B. longum* subsp. *longum* DJO10A and NCC2705 and *B. longum* subsp. *infantis* ATCC 15697), the *B. adolescentis* group (*B. adolescentis* ATCC 15703 and *B. dentium* Bd1), and the *B. animalis* group (*B. animalis* subsp. *lactis* AD011, BI-04, DSM 10140, and Bb-12) (Fig. 2). A whole-genome alignment of these genomes was consistent with this analysis, with *B. animalis* subsp. *lactis* clearly the most distantly related to the other groups. The four strains of *B. animalis* subsp. *lactis* exhibited >99% sequence identity over their entire genomes, illustrating a very closely related group (Fig. 3). Interestingly, in the whole-genome comparison of the *B. longum* group, *B. longum* subsp. *infantis* showed a large inverted region (~1.3 Mb) in the middle of the genome compared to both *B. longum* subsp. *longum* strains, suggesting that this possible inversion is a recent evolutionary event within *B. longum* subsp. *infantis*, or it may be due to errors in genome assembly (Fig. 3). Large genome inversions were previously observed in a genome comparison of strains of *Salmonella enterica*, and they were proposed to be due to homologous recombination between identically repeated rRNA operons (70). However, there are no rRNA operons, mobile elements, or repeat regions in the boundaries of the inversion exhibited in the *B. longum* subsp. *infantis* genome, suggesting a novel inversion mechanism or sequence assembly issues.

### Mobile Elements

Eight insertion sequence (IS) families were found in the nine bifidobacterial genomes, including the IS3, IS21, IS30, IS110, IS150, IS256, IS607/IS200, and ISL3 families (Table 8). While *B. dentium* has the smallest set of IS elements, *B. longum* subsp. *infantis* contains the most IS elements and the most diversity in IS families, displaying all eight of the families. This is consistent with it containing the largest genome among these nine bifidobacterial strains. Interestingly, the three sequenced strains of *B. animalis* subsp. *lactis* contained very few IS elements, and all of these were from a single IS family, ISL3. This family of IS elements was first characterized from the yogurt bacterium *Lactobacillus delbrueckii* subsp. *bulgaricus* (95). Since *B. animalis* subsp. *lactis* is also used frequently in yogurt manufacturing, the presence of complete ISL3 elements (none of which are present in the other bifidobacterial genomes) in this subspecies may suggest horizontal gene transfer between bacteria in the yogurt fermentation environment.

IS elements are frequently involved in genome rearrangement or deletion events (29, 63, 211). During pure culture growth of the intestinal strain *B. longum* subsp. *longum* DJO10A, the IS30 element was found to move within the genome, confirming that it was indeed active (161). This hyperactivity of IS30 was also implicated in a genome deletion event in this strain during its adaptation to pure culture growth, as a large gene cluster encoding lantibiotic production, positioned between IS30 elements, was precisely deleted from

TABLE 6. Completed or ongoing bifidobacterial genome projects

Organism	Strain	Status of project	Sequence availability <sup>a</sup>	Size (Mb) <sup>b</sup>	Institution <sup>c</sup>	Reference or GenBank accession no.
<i>B. adolescentis</i>	ATCC 15703	Complete	Yes	2.09	Gifu University, Japan	AP009256 <sup>d</sup>
	L2-32	Ongoing	Yes	2.39	Washington University, USA	
<i>B. angulatum</i>	DSM 20098	Ongoing	Yes	2.00	Washington University, USA	
	JCM7096	Ongoing	No	2.00	University of Tokyo, Japan	
<i>B. animalis</i> subsp. <i>lactis</i>	AD011	Complete	Yes	1.93	KRIBB, South Korea	141
	Bb-12	Complete	Yes	1.94	Integrated Genomics, Inc./Chr. Hansen, USA	92
	BI-04	Complete	Yes	1.94	Danisco/Penn State University, USA	17
	DSM 10140	Complete	Yes	1.94	Danisco/Penn State University, USA	17
	HN019	Ongoing	Yes	1.92	Fonterra Research Center, New Zealand	
	V9	Ongoing	Yes	1.94	Inner Mongolia Agricultural University, China	
<i>B. bifidum</i>	BGN4	Ongoing	No		KRIBB, South Korea	
	DSM 20456	Ongoing	No		Washington University, USA	
	JCM1255	Ongoing	Yes	2.00	University of Tokyo, Japan	
	NCIMB 41171	Ongoing	Yes	2.19	Broad Institute, USA	
<i>B. breve</i>	DSM 20213	Ongoing	Yes	2.30	Washington University, USA	
	JCM1192	Ongoing	Yes	2.00	University of Tokyo, Japan	
	UCC2003	Ongoing	No	2.42	University College, Cork, Ireland	
<i>B. catenulatum</i>	DSM 16992	Ongoing	Yes	2.06	Washington University, USA	
	JCM1194	Ongoing	Yes	2.00	University of Tokyo, Japan	
<i>B. dentium</i>	ATCC 27678	Ongoing	Yes	2.62	Washington University, USA	335
	ATCC 27679	Ongoing	No		BCM-HGSC, USA	
	Bd1	Complete	Yes	2.64	University of Parma, Italy	
	JCM1195	Ongoing	No		University of Tokyo, Japan	
	JCVIHP022	Ongoing	No		J. Craig Venter Institute, USA	
	JCVIHP023	Ongoing	No		J. Craig Venter Institute, USA	
<i>B. gallicum</i>	DSM 20093	Ongoing	Yes	2.02	Washington University, USA	
<i>B. longum</i> subsp. <i>longum</i>	BORI	Ongoing	No		KRIBB, South Korea	
	DJO10A	Complete	Yes	2.38	JGI/University of Minnesota, USA	161
	NCC2705	Complete	Yes	2.26	Nestle/ University of Georgia, USA	275
	JDM301	Ongoing	Yes	2.48	Shanghai Jiao Tong University, China	
<i>B. longum</i> subsp. <i>infantis</i>	157F-NC	Ongoing	No		University of Tokyo, Japan	280
	ATCC 15697	Complete	Yes	2.83	JGI/UC Davis, USA	
	ATCC 55813	Ongoing	Yes	2.37	BCM-HGSC, USA	
	CCUG 52486	Ongoing	Yes	2.45	Broad Institute, USA	
	JCM1217	Ongoing	No		University of Tokyo, Japan	
	JCM1222	Ongoing	No		University of Tokyo, Japan	
<i>B. pseudocatenulatum</i>	DSM 20438	Ongoing	Yes	2.30	Washington University, USA	
<i>B. scardovii</i>	JCM12489	Ongoing	No		University of Tokyo, Japan	
<i>Bifidobacterium</i> sp.	12_1_47BFAA	Ongoing	No		Broad Institute, USA	
	HM5	Ongoing	No		University of Tokyo, Japan	
	JCM15439	Ongoing	No		University of Tokyo, Japan	

<sup>a</sup> Complete or draft genome sequences are available in the GenBank database and the Human Metagenome Consortium Japan (HMCJ) database.

<sup>b</sup> Approximate size.

<sup>c</sup> KRIBB, Korea Research Institute of Bioscience & Biotechnology; BCM-HGSC, Baylor College of Medicine-Human Genome Sequencing Center; JGI, Joint Genome Institute, U.S. Department of Energy.

<sup>d</sup> Published in GenBank only.

the genome. This supports the role of IS elements in genome reduction and rapid genome adaptation to new environments.

The genome sequences of both *B. longum* subsp. *longum* strains revealed novel mobile integrase cassette (MIC) structures, consisting of three contiguous integrases flanked by an inverted repeat and a palindrome structure sandwiched by two

IS3-type IS elements, and their nonlinear positioning within the genomes suggests that they are active (161). Interestingly, these MIC structures are not present in other bifidobacterial genomes, including that of *B. longum* subsp. *infantis*, or in other intestinal bacterial genomes, suggesting that this MIC structure is specific to *B. longum* subsp. *longum* (161). During



TABLE 7. General characteristics of bifidobacterial genomes

Parameter	Value									
	<i>B. adolescentis</i> ATCC 15703	<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. dentium</i> Bd1	AD011	<i>B. animalis</i> subsp. <i>lactis</i>			
		DJO10A	NCC2705				BI-04	DSM 10140	Bb-12	
Length (bp)	2,089,645	2,375,792	2,256,640	2,832,748	2,636,367	1,933,695	1,938,709	1,938,483	1,942,198	
Overall G + C content (%)	59.18	60.15	60.12	59.86	58.94	60.49	60.48	60.48	60.48	
No. of plasmids	0	2	1	0	0	0	0	0	0	
No. of annotated genes	1,631	1,990	1,727	2,416	2,129	1,528	1,567	1,566	1,642	
Avg gene length (bp)	1,109	1,031	1,115	997	1,066	1,070	1,064	1,062	1,063	
Gene density	0.780	0.838	0.765	0.852	0.807	0.790	0.808	0.807	0.845	
(no. of genes/kb)										
Gene coding content (%)	86.5	86.4	85.3	85.1	86.1	84.6	86.0	85.8	89.9	
Gene G + C content (%)	60.08	61.13	60.86	60.70	59.32	61.47	61.40	61.40	61.11	
No. of rRNA gene operons	5	4	4	4	4 (+ 1 5S rRNA gene)	2 (+ 1 5S rRNA gene)	4	4	4	
No. of prophage-like elements	0	1	1 (remnant)	4	1 complete + 1 remnant	1 (remnant)	1 (remnant)	1 (remnant)	1 (remnant)	
No. of tRNAs	54	58	57	79	55	52	52	52	52	
No. of tRNA synthetases	20	20	21	19	19	21	19	19	19	
No. of MICs	0	4	3	0	0	0	0	0	0	
No. of CRISPRs	1 (86 repeats)	1 (43 repeats)	0	0	2 (17 and 81 repeats)	1 (20 repeats)	1 (22 repeats)	1 (20 repeats)	1 (20 repeats)	
No. of oligonucleotide clusters <sup>a</sup>	10	11	7	11	10	7	7	7	7	
No. of polycl clusters <sup>b</sup>	1	1	0	0	1	0	0	0	0	

<sup>a</sup> Number of predicted oligosaccharide and other complex carbohydrate utilization gene clusters.

<sup>b</sup> Number of predicted polyclonal utilization gene clusters.

pure culture adaptation of *B. longum* subsp. *longum* strain DJO10A over 1,000 generations, a MIC element was found to be deleted from the genome along with ~50 kb of sequence, indicating that these structures can play a role in deletion events (161). This is further substantiated by a predicted deletion from the genome of *B. longum* subsp. *longum* strain NCC2705 of a frameshifted *tetW* gene and bordering MIC element that are present in strain DJO10A (161).

tRNAs and Aminoacyl-tRNA Synthetases

Even though all nine sequenced bifidobacterial genomes encode multiple tRNAs for all 20 amino acids, they do not encode aminoacyl-tRNA synthetases for asparagine and glutamine, suggesting that they may utilize alternative pathways for translation for these amino acids, as has been shown for some other bacteria (187, 294). These alternative pathways utilize GltX (glutamyl-tRNA synthetase) and AspS (aspartyl-tRNA synthetase) for the synthesis of intermediates (Glu-tRNA<sup>Gln</sup> and Asp-tRNA<sup>Asn</sup>) and GatABC (Glu-tRNA<sup>Gln</sup>/Asp-tRNA<sup>Asn</sup> amidotransferases) and then convert these intermediates to Gln-tRNA<sup>Gln</sup> and Asn-tRNA<sup>Asn</sup> for incorporation of these amino acids during translation. Genes encoding GltX, AspS, and GatABC are present in all sequenced bifidobacterial genomes, supporting the utilization of these alternative translation pathways in bifidobacteria (161, 275, 335). Interestingly, the predicted alanyl-tRNA synthetase (*AlaS*) gene in *B. animalis* subsp. *lactis* AD011 is longer than those in the other three *B. animalis* subsp. *lactis* genomes because of the presence of two identical 378-bp repeat regions in the gene, which would result in a frameshift, preventing expression of a full alanyl-tRNA synthetase. A perfect sequence match of the *alaS* gene in strain AD011, after removal of one of the repeat regions, to the *alaS* genes in the three other *B. animalis* subsp. *lactis* genomes suggests that this repeat is probably a genome assembly error.

Prophages

Prophage-like elements were found in eight of the nine complete bifidobacterial genomes (with the *B. adolescentis* genome being the exception), with the majority being incomplete (Table 7). An incomplete prophage-like element from *B. breve* UCC2003 has also been reported (332). Recently, a comparative analysis of complete and partial prophage-like elements in three complete bifidobacterial genomes (*B. dentium* Bd1, *B. longum* subsp. *infantis* ATCC 15697, and *B. animalis* subsp. *lactis* AD011), as well as 11 elements in six draft bifidobacterial genomes (*B. dentium* ATCC 27678, *B. longum* subsp. *infantis* CCUG 52486, *B. bifidum* 317B and NCIMB 41171, *B. adolescentis* L2-32, and *B. catenulatum* DSM 16992), provided more insights into the taxonomy and evolution of bifidobacterial prophages (334).

It was demonstrated previously that three prophages, in *B. longum* subsp. *longum* DJO10A, *B. longum* subsp. *infantis* ATCC 15697 (Binf-4), and *B. animalis* subsp. *lactis* AD011, are inducible at the transcription level by use of hydrogen peroxide. One prophage in *B. dentium* Bd1 (Bdent-2) could also be induced at the transcription level by culture in basal medium mucin. None could be demonstrated to complete a full lytic

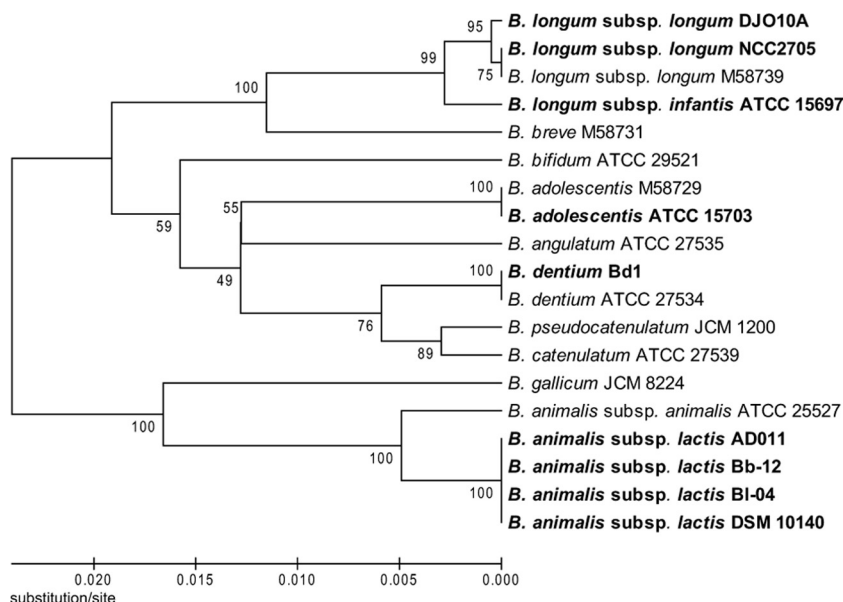


FIG. 2. Phylogenetic analysis of the 16S rRNA genes of the nine sequenced bifidobacteria, illustrating their separation into three distinct groups. The 16S rRNA gene sequences from complete or draft bifidobacterial genomes were compared by ClustalW multiple sequence alignments as described in the legend to Fig. 1. The completely sequenced bifidobacteria are indicated in bold.

cycle, suggesting that there is a novel signal required for full induction or that they may not be 100% complete (332, 334). It has also been suggested that full induction of the lytic cycle may require a helper phage (334). A microarray analysis of *B. dentium* Bd1 (Bdent-2) revealed some genes that are known to be required for a lytic lifestyle, but not all were expressed, thus explaining why this prophage DNA sequence cannot produce viable phage particles (334). The prophage in *B. longum* subsp. *longum* DJO10A contains 57 genes in a 37.8-kb cluster, and its insertion site has highly conserved direct repeats, representing a likely duplication of its attachment site during its insertion. This attachment site is located in the 3' region of a tRNA<sup>Met</sup> gene, resulting in the prophage insertion occurring directly downstream from this gene. Interestingly, an identical insertion site is frequently found in other bifidobacterial prophage-like elements, suggesting that this attachment site is utilized as a general prophage insertion mechanism (332, 334). The insertion of prophages and other DNA elements utilizing certain tRNA genes has been observed in other bacteria, and this attachment site appears to be a favorable insertion position (34). While the prophage-like elements in *B. longum* subsp. *infantis* and the three strains of *B. animalis* subsp. *lactis* are not inserted bordering this tRNA gene, other DNA elements, possibly of plasmid origin, are inserted in this region, substantiating the hypothesis that certain tRNA genes provide hot spots for insertion of foreign DNA elements.

Comparative sequence analysis of prophage-like elements in bifidobacteria indicated that they can be divided into four groups, with the majority belonging to group II, which is related to the low-GC *Firmicutes*, such as *Lactococcus* and *Lactobacillus*. A number of them also encode products not required for phage functions, such as type I or type II restriction-modification (R-M) systems or metabolic factors such as polyketide biosynthesis enzymes and phosphofructokinase

(334). The presence of these extra functions may contribute to the overall cellular advantage of acquiring these elements and also may benefit the ecological fitness of the lysogeny. The prophage present in *B. longum* subsp. *longum* DJO10A and one of the prophages (Binf-4) present in *B. longum* subsp. *infantis* carry a tRNA<sup>Ser</sup> and a tRNA<sup>Trp</sup> gene, respectively (161, 334). The tRNA<sup>Ser</sup> gene in the strain DJO10A prophage recognizes the most common Ser codon in the prophage genome, while it is not a common Ser codon in the genome of this strain, suggesting that this was the selective pressure for the prophage acquiring this tRNA gene. This was not the case for the *B. longum* subsp. *infantis* prophage, as tRNA<sup>Trp</sup> recognizes only one codon. Since the frequencies of the Trp codon are similar between the prophage and the bacterial genome, this suggests that the acquisition may be of mutual benefit to both the prophage and the bacterium.

Diversity-generating retroelement (DGR) structures have been detected in some bacteriophages, and they are proposed to be involved in a mechanism to rapidly evolve phage tail proteins, which can potentially expand the host range for the phage (79). These structures are composed of genes encoding reverse transcriptase and a tail protein and two direct repeat sequences, one an invariable template repeat (TR) located in the intergenic region between the two genes and the other a variable repeat (VR) located in the 3' region of the tail protein gene. The TR is proposed to be reverse transcribed, which produces a single-stranded DNA copy with nucleotide substitutions only at adenine residues, and this cDNA switches with the VR region in an event termed "mutagenic homing" (32). Two bifidobacterial prophages (located in *B. longum* subsp. *longum* DJO10A and *B. adolescentis* L2-32) carry DGR structures consisting of putative genes encoding reverse transcriptase and a tail protein and direct repeat sequences consistent with TR and VR regions (332, 334). The only mismatches between the TR and

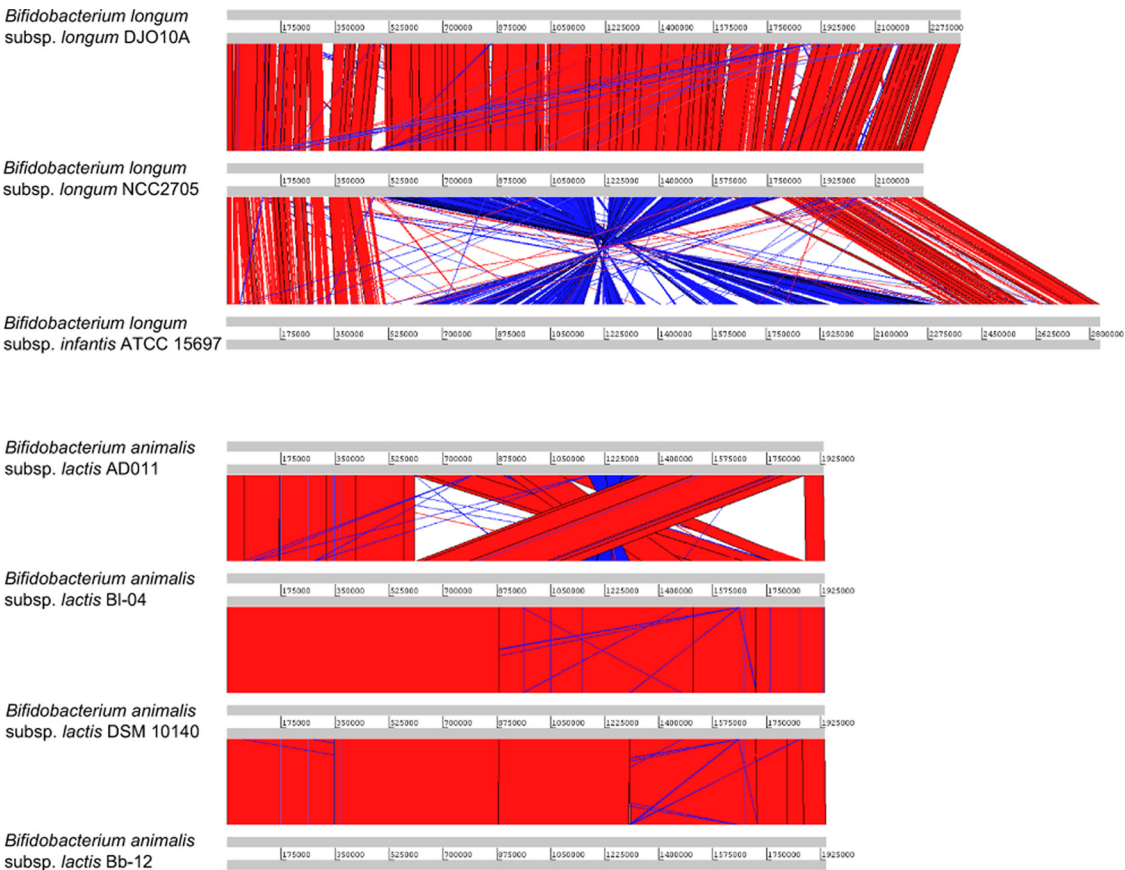


FIG. 3. Alignments of the complete genome sequences of the *B. longum* group and the *B. animalis* subsp. *lactis* group. Red lines indicate the relative locations of elements that are oriented in the same direction. Blue lines indicate elements orientated in opposite directions.

VR sequences in strain DJO10A are located at 13 adenine residues in TR that do not match with VR, consistent with the proposed function of these mutator elements.

CRISPR Structures in Bifidobacteria

CRISPRs are DNA repeats which have been found widely, in more than 40% of bacterial genomes and most archaeal genomes (103, 129, 155). CRISPR structures consist of identically repeated short (24 to 47 bases) DNA sequences interspersed with sequence-variable spacer regions, varying in length from 26 to 72 bases, and with CRISPR-associated *cas*

genes (33, 110, 122, 296). The number of repeat sequences is variable between different bacteria, ranging from as few as 2 to as many as 249 (296). This structure was found in seven of the nine bifidobacterial genomes, with those of *B. longum* subsp. *longum* NCC2705 and *B. longum* subsp. *infantis* being the exceptions (Table 7). CRISPRs have been demonstrated to be involved in acquiring resistance against infecting bacteriophages by replacing the first spacer sequence with a DNA segment originating from bacteriophage DNA (18, 71). In addition to the completely sequenced bifidobacteria, these structures can also be seen in the draft sequences of *B.*

TABLE 8. IS elements in bifidobacteria

IS family	No. of elements (no. of complete elements) <sup>a</sup>								
	<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	<i>B. animalis</i> subsp. <i>lactis</i>			
	DJO10A	NCC2705				AD011	BI-04	DSM 10140	Bb-12
IS3	13 (1)	14 (2)	16 (9)	8 (3)	6 (5)	0	0	0	0
IS21	10 (8)	7 (5)	7 (1)	5 (3)	0	0	0	0	0
IS30	9 (5)	5 (3)	15 (2)	2 (0)	0	0	0	0	0
IS110	0	0	<b>6 (5)</b>	0	0	0	0	0	0
IS256	4 (2)	7 (5)	7 (2)	3 (1)	0	0	0	0	0
IS607/IS200	1 (1)	1 (1)	2 (0)	0	0	0	0	0	0
ISL3	7 (0)	12 (0)	3 (0)	0	0	<b>7 (6)</b>	<b>6 (5)</b>	<b>6 (5)</b>	<b>6 (6)</b>

<sup>a</sup> Numbers in bold indicate species-specific IS elements.

TABLE 9. Comparison of CRISPR-cas systems in bifidobacteria

Species	No. of repeats	Group <sup>a</sup>	Repeat sequence	Structure <sup>b</sup>
<i>B. longum</i> subsp. <i>longum</i> DJO10A	43	I	CAAGCTTATCAAGAAGGGTGAATGCTAATT CCCAGC	<i>c5-c1-c2</i> -repeats
<i>B. dentium</i> Bd1	17	I	CAAGTTTATCAAGAAGGGTAGAAGCTAATT CCCAGT	<i>csn1-c1-c2</i> -repeats
	81	II	GTCGCTCTCCTCACGGAGAGCGTGGATTG AAAT	<i>c3-c5-csd1-c1</i> -repeats
<i>B. dentium</i> ATCC 27678	15	I	CAAGTTTATCAAGAAGGGTAGAAGCTAATT CCCAGT	<i>c5-c1-c2</i> -repeats
	19	II	GTCGCTCTCCTCACGGAGAGCGTGGATTG AAAT	<i>c3-c5-csd1-csd2-c4-c1-c2</i> -repeats
<i>B. adolescentis</i> ATCC 15703	86	II	GGTCGCTCTCCTTACGGAGAGCGTGGATTG AAAT	<i>c3-c5-csd1a-csd1b-csd2-c4-c1-c2</i> -repeats
<i>B. adolescentis</i> L2-32B	113	II	GGTCGCTCTCCTTACGGAGAGCGTGGATTG AAAT	<i>c3-csd2-c4-c1-c2</i> -repeats 2-repeats 1
<i>B. animalis</i> subsp. <i>lactis</i> AD011	20	III	CCCTCAATGAAGCTCCGAAGCCGAGACTTC GGAGAT	<i>c3-c_GSU0053-c1</i> -repeats
<i>B. animalis</i> subsp. <i>lactis</i> HN019	20	III	CCCTCAATGAAGCTCCGAAGCCGAGACTTC GGAGAT	<i>c3-hyp-c_GSU0053-c2-c1</i> -repeats
<i>B. animalis</i> subsp. <i>lactis</i> BI-04	22	III	CCCTCAATGAAGCTCCGAAGCCGAGACTTC GGAGAT	<i>csb3-c3-csb2-csb1-c2-c1</i> -repeats
<i>B. animalis</i> subsp. <i>lactis</i> DSM 10140	20	III	CCCTCAATGAAGCTCCGAAGCCGAGACTTC GGAGAT	<i>csb3-c3-csb2-csb1-c2-c1</i> -repeats
<i>B. animalis</i> subsp. <i>lactis</i> Bb-12	20	III	CCCTCAATGAAGCTCCGAAGCCGAGACTTC GGAGAT	<i>csb3-c3-csb2-csb1-hyp-c1</i> -repeats
<i>B. angulatum</i> DSM 20098	139?	IV	GTGTTCCCCGCACACGCGGGGATGATCCC	<i>c3-cse1-cse2-cse4-c5-cse3-c1-c2</i> -repeats
<i>B. catenulatum</i> JCM1194	34	IV	GTGTTCCCCGCATACGCGGGGATGATCCC	<i>c3-truncated-cse4-c5-cse3-c1-c2</i> -repeats

<sup>a</sup> CRISPR groups represent elements with identical repeat sequences.

<sup>b</sup> *c1* to *c5*, CRISPR-associated *cas1* to *cas5* genes; *csd1-csd2*, CRISPR-associated *csd1-csd2* family genes; *c\_GSU0053*, GSU0053 family CRISPR-associated gene; *cse1* to *cse4*, CRISPR-associated *cse1* to *cse4* family genes; *csn1*, CRISPR-associated *csn1* family gene; *hyp*, hypothetical protein gene; truncated, incomplete draft sequence.

*dentium* ATCC 27678, *B. adolescentis* L2-32, *B. animalis* subsp. *lactis* HN019, *B. angulatum* DSM 20098, and *B. catenulatum* JCM1194 (Table 9). Interestingly, one of the two CRISPR structures in the *B. dentium* strain contains a repeat sequence identical to the CRISPR structure in *B. longum* subsp. *longum* DJO10A, suggesting a common origin for the CRISPR structures between these two strains. Given their widespread presence in bifidobacteria, these structures may be due to selective pressure to protect these bacteria from bacteriophages in the large intestine. Further substantiation of a functional role for these structures comes from an analysis of bifidobacterial prophages that found 29 regions with >85% sequence identity between 12 prophages and 21 CRISPR spacers (334).

Since the CRISPR model for protecting against invading bacteriophages involves replacing a spacer region with part of the invading phage DNA, a search for DNA regions identical to spacer regions found several extrachromosomal DNA elements containing these sequences (33, 191). This suggests an expanded role for these structures in protection against all invading DNA elements. Analysis of spacers in bifidobacterial CRISPR structures revealed that there is one 28-bp spacer in *B. longum* subsp. *longum* DJO10A (spacer number 5) that has 100% sequence identity with an internal sequence in the putative *mob* genes carried by the bifidobacterial plasmids p6043B, pNAC2, and pMG1. This further supports an expanded role for CRISPR structures.

### Chromosome Origin of Replication

The origin of replication (*oriC*) in bacteria is generally highly conserved within species, and generally there is more than one *oriC* cluster per genome (170). Each cluster contains an *oriC* and multiple DnaA boxes for the binding of DNA polymerase. The origin of replication (*oriC*) in the *B. longum* subsp. *longum* NCC2705 genome was not initially predicted by a total GC skew analysis due to initial errors with genome sequence assembly (105). Geometric analysis for the assembly of whole genome sequences showed that the genome sequence of *B. longum* NCC2705 was misassembled because of misplacement of several repeated regions. Following a correction of these assembly errors in the revised genome sequence of *B. longum* NCC2705 (275), the highly conserved *oriC* region was revealed by bioinformatic predictions. The *oriC*-associated regions of seven complete genome sequences of bifidobacteria also showed the same organization, with three *oriC* clusters and seven different kinds of putative DnaA boxes in highly conserved locations, as follows: *parB-parA*-(*oriC* cluster 3)-*gidB-ssb-oxaA-rnpA-rpmH*-(*oriC* cluster 2)-*dnaA*-(*oriC* cluster 1)-*dnaN-recF-hyp-gyrB-gyrA*. The *oriC* clusters contain the same number and type of DnaA boxes for each member of a species, but the number and type are species specific (Table 10). The locations of the three *oriC* clusters in all bifidobacterial genomes are positioned identically within the gene cluster involved in DNA replication. The one apparent exception is *B.*



TABLE 10. Comparison of DnaA boxes in nine bifidobacterial genomes

Strain	<i>oriC</i> cluster <sup>a</sup>	No. of DnaA boxes <sup>b</sup>							Total no. of DnaA boxes
		A	B	C	D	E	F	G	
<i>B. longum</i> subsp. <i>longum</i> DJO10A	1	3	1	3	1	0	0	0	8
	2	1	1	0	0	1	1	0	4
	3	6	0	0	0	0	0	1	7
	Subtotal	10	2	3	1	1	1	1	19
<i>B. longum</i> subsp. <i>longum</i> NCC2705	1	3	1	3	1	0	0	0	8
	2	1	1	0	0	1	1	0	4
	3	6	0	0	0	0	0	1	7
	Subtotal	10	2	3	1	1	1	1	19
<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	1	3	1	3	1	0	0	0	8
	2	1	0	0	0	1	1	0	3
	3	5	0	0	0	0	0	0	5
	Subtotal	9	1	3	1	1	1	0	16
<i>B. adolescentis</i> ATCC 15703	1	5	0	2	0	0	1	0	8
	2	1	0	0	0	1	1	0	3
	3	4	0	0	0	1	0	0	5
	Subtotal	10	0	2	1	2	2	0	17
<i>B. dentium</i> Bd1	1	5	0	1	1	1	0	1	9
	2	1	0	0	0	1	1	0	3
	3	2	1	0	0	1	0	0	4
	Subtotal	8	1	1	1	3	1	1	16
<i>B. animalis</i> subsp. <i>lactis</i> AD011	1	6	0	1	0	1	1	0	9
<i>B. animalis</i> subsp. <i>lactis</i> BI-04	2	1	0	0	1	0	0	0	2
<i>B. animalis</i> subsp. <i>lactis</i> DSM 10140	3	3	1	0	0	1	0	0	5
<i>B. animalis</i> subsp. <i>lactis</i> Bb-12	Subtotal	10	1	1	1	2	1	0	16

<sup>a</sup> Locations of *oriC* clusters in *oriC*-associated conserved regions are as follows: *parB-parA-(oriC cluster 3)-gidB-ssb-oxaA-rnpA-rpmH-(oriC cluster 2)-dnaA-(oriC cluster 1)-dnaN-recF-hyp-gyrB-gyrA*.

<sup>b</sup> The DnaA boxes consist of seven types, designated A to G, as follows: A, TTATCCACA; B, TTGTCCACA; C, TTTTCCACA; D, TTACCCACA; E, TTATCCACC; F, TTATTCACA; and G, TTATGCACA.

*longum* subsp. *infantis* ATCC 15697, whose annotation does not show an *rmpH* gene, which encodes the 50S ribosomal protein L34. However, analysis of its sequence shows that the gene is present, indicating that the open reading frame (ORF) was missed during genome annotation.

### R-M Systems

R-M systems play an important role in protection of methylated host DNA against invasion of unmethylated foreign DNA, such as plasmids or bacteriophages (236). An analysis of R-M systems in the nine bifidobacterial genomes revealed that only *B. longum* subsp. *longum* genomes have type I R-M systems and that all genomes except for that of *B. longum* subsp. *infantis* ATCC 15697 have one or two type II R-M systems (Table 11). The lack of any observed restriction-modification system or CRISPR structure in the *B. longum* subsp. *infantis* ATCC 15697 genome suggests that it may be more susceptible to phage infection. This is supported by the increased number of prophages in its genome, which is four times that for any of the other genomes (Table 7). The type II R-M systems encoded by the bifidobacterial genomes are analogs of Sau3AI, EcoRII, EcoRI, and KpnI (Table 11). It is intriguing that *B. adolescentis* carries gene analogs for the two restriction subunits of the multimeric LlaJI restriction enzyme but is missing genes analogous to those for the methylase subunits. This

restriction enzyme is encoded by genes for the R1.LlaJI subunit, which recognizes the restriction site; R2.LlaJI, which is required to enable restriction of the DNA; and two methylase subunits (208). The absence of the gene analogs for the methylases suggests that the genes may not be expressed or have developed another function. The positioning of an IS3-type IS element directly upstream from these genes may suggest its role in a deletion event. Since genes comprising a KpnI gene cluster are located directly downstream, this may suggest an evolutionary connection to the formation of unusual multimeric restriction enzymes such as LlaJI. Both of these restriction enzymes are protected by 5 methylcytosines within their recognition sites, and while there are similarities within their recognition sites, they are not identical. Interestingly, the four strains of *B. animalis* subsp. *lactis* encode only one R-M system, which is analogous to an EcoRI-type system and is positioned in a predicted integrated plasmid with a significantly (10%) lower G+C content than that of its genome, suggesting that it was acquired from a recent horizontal gene transfer event. Given that bacteriophage infection is very prevalent in dairy fermentation environments (76, 183) and that this subspecies is predicted to have evolved in this environment, these observations suggest that the environment may have provided the selective pressure for acquiring this R-M system.

The genes encoding the R-M systems described above are frequently found in bifidobacterial genomes, and they are among

TABLE 11. R-M systems in nine complete genomes of bifidobacteria

R-M system or analog	Locus tag <sup>a</sup>					
	<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	<i>B. animalis</i> subsp. <i>lactis</i> AD011, BI-04, DSM 10140, and Bb-12
	DJO10A	NCC2705				
Type I systems						
Mrr	BLD_1958	BL1781	ND	ND	BDP_0997	ND
HsdM	BLD_1959	BL1782	ND	ND	ND	ND
HsdS	BLD_1960	BL1783	ND	ND	ND	ND
HsdR	BLD_1962	BL1785	ND	ND	BDP_1319	ND
Type II analogs <sup>b</sup>						
Sau3AI-R	BLD_1358	BL0563	ND	BAD_1232	ND	ND
Sau3AI-M	BLD_1359	BL0564	ND	BAD_1233	ND	ND
EcoRII-R	BLD_0355	BL1472	ND	ND	ND	ND
EcoRII-M	BLD_0356	BL1473	ND	ND	ND	ND
KpnI-R	ND	ND	ND	BAD_1282	ND	ND
KpnI-M	ND	ND	ND	BAD_1283	ND	ND
EcoRI-R	ND	ND	Blon_1196 (truncated) <sup>c</sup>	ND	ND	BLA_0644, Balac_1446, Balat_1446, BIF_00872
EcoRI-M	ND	ND	Blon_1197	ND	ND	BLA_0645, Balac_1447, Balat_1447, BIF_01894
R1.LlaII	ND	ND	ND	BAD_1280	ND	ND
R2.LlaII	ND	ND	ND	BAD_1281	ND	ND

<sup>a</sup> ND, not detected.<sup>b</sup> R and M represent restriction enzymes and methyltransferases for type II R-M systems, respectively.<sup>c</sup> This pseudogene is truncated by insertion of an IS element.

the major factors inhibiting the introduction of foreign DNA into these bacteria, resulting in very low electroporation frequencies. To overcome this obstacle, type II methylase genes from *B. adolescentis* and *B. breve* were expressed in *E. coli* to enable the methylation of *E. coli*-*Bifidobacterium* shuttle vectors (195, 348). This greatly improved transformation efficiencies, to a level that permitted targeted mutagenesis in bifidobacteria (195).

### Biosynthesis of Amino Acids, Vitamins, Purines, and Pyrimidines

Although bifidobacteria have been studied for over 1 century, the lack of genetic information has limited insights into their biosynthetic capabilities. While some biosynthetic capabilities for vitamins and amino acids were elucidated previously (25, 244, 269), the genome analysis of bifidobacteria substantiates these biosynthetic capabilities as well as uncovering all other potential biosynthetic capabilities (275). The bifidobacterial genomes have genes predicted to be involved in the biosynthesis of all 20 amino acids, but since bifidobacteria cannot grow in minimum media, it is likely that not all are functional. It can be predicted from the genome analysis that amino acids can potentially be synthesized from different intermediates. These include ribose-5-phosphate (for histidine), glycerate-3-phosphate (for serine, glycine, and threonine), phosphoenolpyruvate (PEP) (for tyrosine, tryptophan, and phenylalanine), pyruvate (for valine, leucine, isoleucine, and alanine), oxaloacetate (for aspartate, asparagine, arginine, lysine, methionine, and cysteine), and 2-oxoglutarate (for glutamate, glutamine, and proline) (Fig. 4). However, since some core genes involved in sulfur assimilation (ATP sulfurylase, adenosine-5'-phosphosulfate kinase, serine acetyltransferase, and cysteine synthetase) are missing, synthesis of cysteine and methionine could still potentially be achieved in the presence

of hydrogen sulfide (H<sub>2</sub>S), which in the large intestine could be supplied by other intestinal bacteria (301). The H<sub>2</sub>S produced by the intestinal flora may be assimilated with *O*-succinyl L-homoserine by cystathionine  $\gamma$ -synthase (EC 2.5.1.48) to produce cystathionine, and then cysteine and methionine biosynthesis could potentially be completed with cystathionine  $\gamma$ -lyase (EC 4.4.1.1), cystathionine  $\beta$ -lyase (EC 4.4.1.8), and methionine synthase (EC 2.1.1.14) in bifidobacteria (Fig. 5).

Previous studies of vitamin biosynthesis by bifidobacteria showed that *B. bifidum*, *B. breve*, *B. adolescentis*, *B. longum* subsp. *infantis*, and *B. longum* subsp. *longum* can produce the vitamins nicotinate, thiamine (B<sub>1</sub>), pyridoxine (B<sub>6</sub>), folate (B<sub>9</sub>), and cobalamin (B<sub>12</sub>) (67). Analysis of the nine sequenced bifidobacterial genomes showed that they all contain gene analogs associated with the biosynthesis of nicotinate, thiamine (B<sub>1</sub>), and folate (B<sub>9</sub>), but they do not carry genes for biosynthesis of cobalamin (B<sub>12</sub>). This may suggest that production of these vitamins in bifidobacteria occurs by other enzymes, or it may be due to strain differences between the strains used in the previous studies and the sequenced strains. Species and/or strain differences in pyridoxine biosynthesis may also exist, as genes encoding three potential proteins (pyridoxine kinase, PdxT, and PdxS) of the pathway were predicted from the three *B. longum* genomes. It has also been shown that bifidobacteria have an absolute requirement for riboflavin (B<sub>2</sub>) (67), pantothenate (B<sub>5</sub>) (113, 308), and biotin (B<sub>7</sub>) (107). While seven of the completed genome sequences for bifidobacteria do not contain genes encoding riboflavin biosynthesis enzymes, two of the genomes, *B. longum* subsp. *infantis* ATCC 15697 and *B. dentium* Bd1, carry the genes for the complete riboflavin biosynthesis pathway (280, 335). This may suggest species and strain differences in riboflavin production in bifidobacteria, as five different strains of *B. longum* subsp. *infantis* were previously shown to require riboflavin (67).

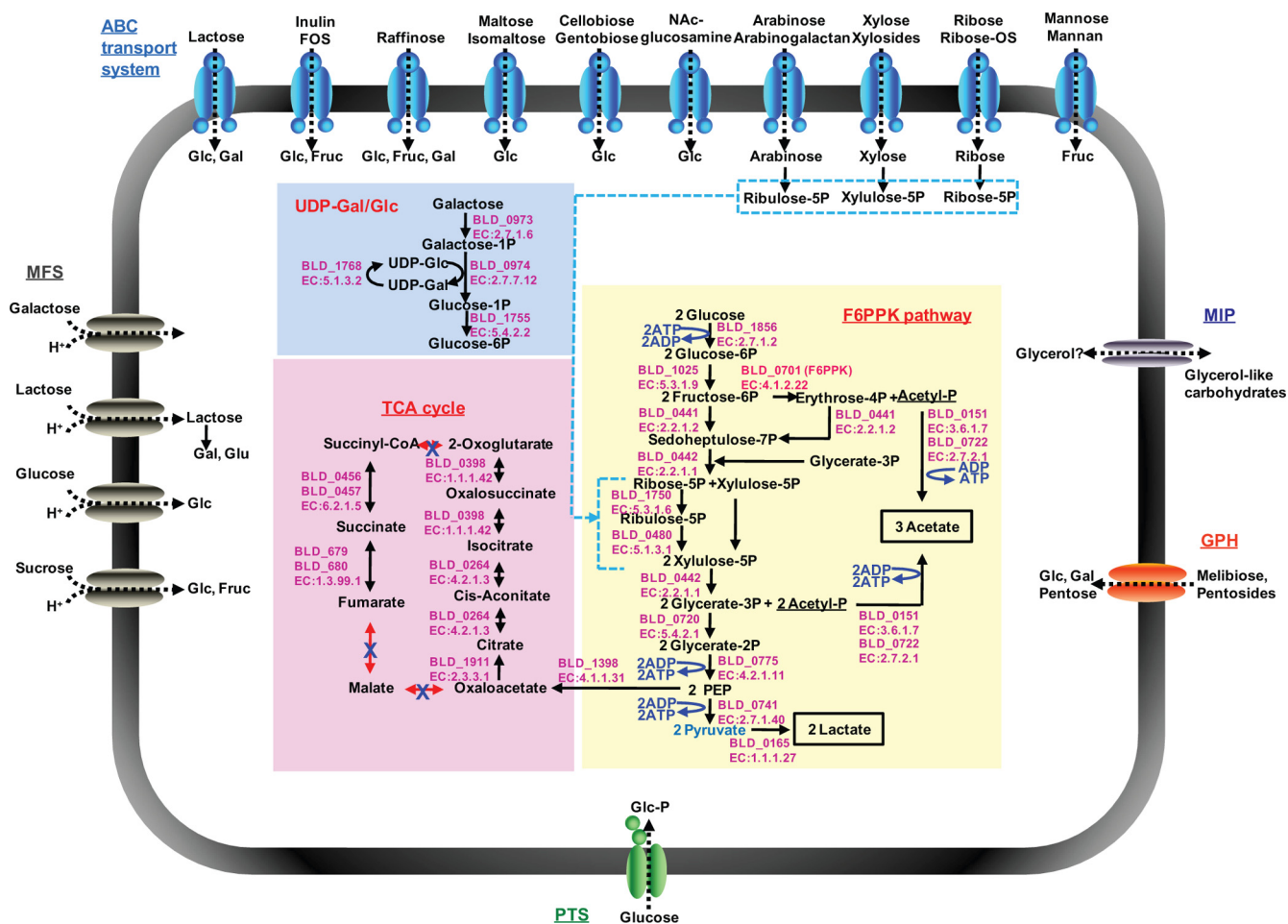


FIG. 4. Overview of predicted carbohydrate uptake and metabolism systems in bifidobacteria. The F6PPK pathway, partial TCA cycle, and UDP-glucose/galactose system (UDP-Glc/Gal) are indicated with different background colors (light yellow for F6PPK, light purple for the partial TCA cycle, and sky blue for the UDP-Glc/Gal system). Blue, ABC transport systems; green, PTS; gray, MFS family; dark purple, major intrinsic protein (MIP) family; orange, GPH cation symporter family. Genes encoding predicted metabolic enzymes from *B. longum* subsp. *longum* DJO10A are indicated.

All nine bifidobacterial genomes carry analogs of all the required genes for biosynthesis of purines and pyrimidines. Surprisingly, a few genes, such as *pyrE*, *pyrF*, and *pyrD*, are duplicated in both sequenced genomes of *B. longum* subsp. *longum*, which is not usual for other bacterial genomes (275). These duplications have been suggested to be involved in the evolution of functionally separate genes in bacteria into a multifunctional gene in higher organisms through a process of gene duplication and DNA rearrangement, leading to in-frame gene fusions encoding chimeric proteins, such as dihydroorotate synthase and UMP synthase in mammalian cells (65).

### Peptidoglycan Biosynthesis

The cell wall structure of bifidobacteria consists of *N*-acetyl-D-glucosamine, muramic acid, ornithine, aspartate, glutamate, alanine, and serine, with a ratio of 1:1:1:1:3:2:1 (322). While *N*-acetyl-D-glucosamine was found to be a growth factor for bifidobacteria (100), they have the capacity to grow without it. In addition, studies have shown that L-rhamnose is a component of bifidobacterial cell walls (200). Eight of the bifidobacterial genomes carry gene analogs for the complete peptidoglycan bio-

synthesis pathway as well as the L-rhamnose biosynthesis pathway, which produces dTDP-rhamnose for cell wall biosynthesis (Table 12). However, the genome sequence of *B. longum* subsp. *infantis* ATCC 15697 does not reveal analogs for two genes involved in the biosynthesis of dTDP-rhamnose, i.e., *rfbA* and *rfbCD*. Interestingly, it contains an analog of *rfbB*, which is arranged in an apparent operon with the other *rfb* genes in the other eight genomes. This may reflect a deletion event in this strain or, possibly, a sequencing error. In addition, although their DNA sequences were present in the genome sequence of *B. animalis* subsp. *lactis* Bb-12, the gene prediction and annotation data did not show *murA* and *murG* genes, which are essential genes for peptidoglycan biosynthesis, indicating possible gene prediction and annotation errors in this genome (Table 12).

### EPS Production

EPS are produced by numerous bacteria, including LAB and bifidobacteria (265). Bacterial EPS can be either homopolysaccharides containing only one sugar monomer or heteropolysaccharides containing a few or several types of sugar monomers

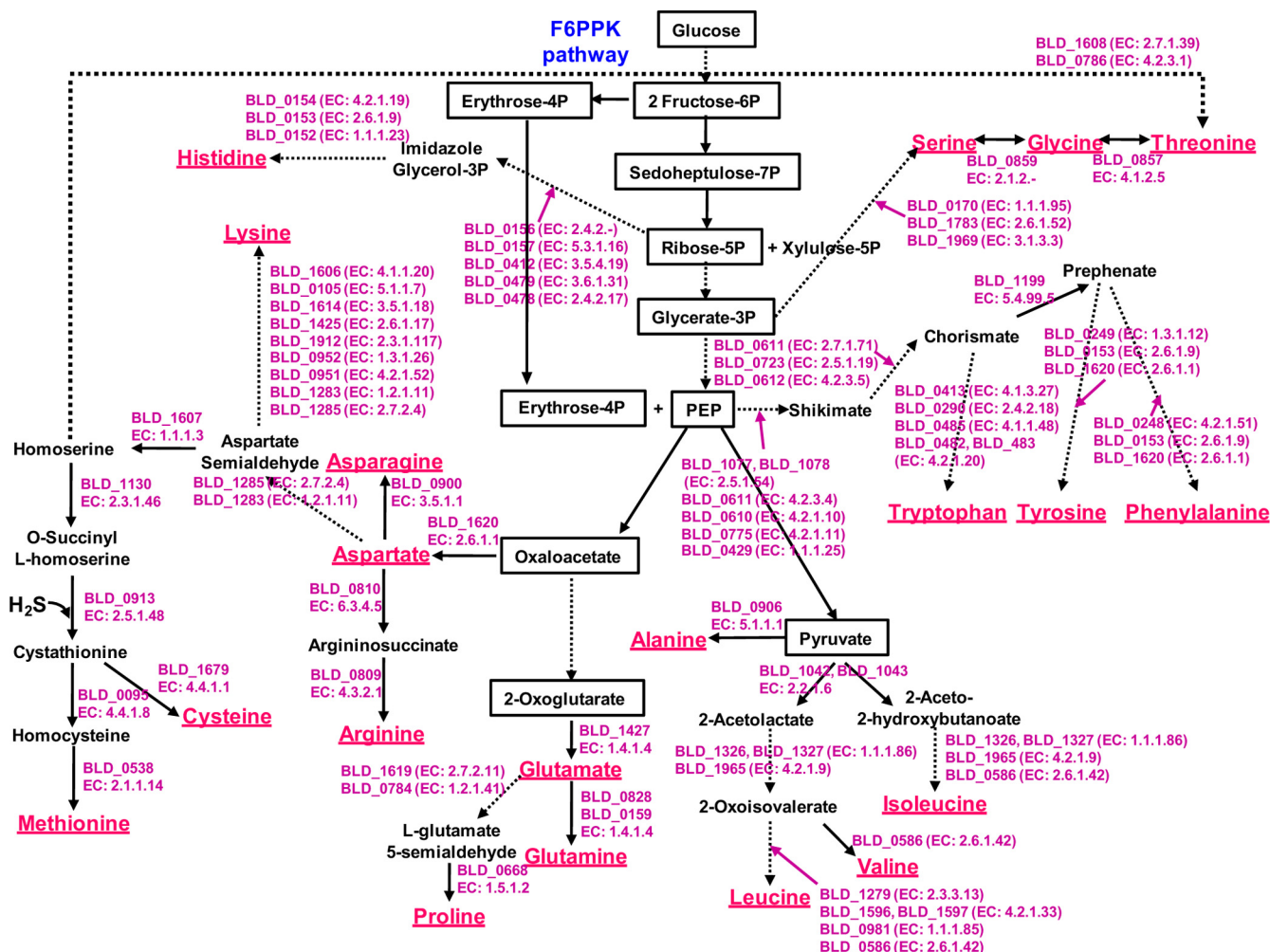


FIG. 5. Predicted amino acid biosynthesis pathways in bifidobacteria. The biosynthesis of amino acids in bifidobacteria utilizes the intermediate products of the F6PPK pathway. Genes predicted to be involved in amino acid biosynthesis in *B. longum* subsp. *longum* DJO10A and the EC numbers of their predicted proteins are indicated in purple. Solid arrows indicate single-step reactions, and dotted arrows indicate multistep reactions.

(35). Most LAB produce heteropolysaccharides consisting of glucose, galactose, and rhamnose, generally at a 2:1.3:1 ratio (201, 243, 261, 320). While EPS production in various LAB has been studied extensively, there are very few studies on EPS production in bifidobacteria. Analysis of the EPS produced by *B. longum* BB-79 revealed that it consisted of galactose and another hexose with lactic acid (250), suggesting that it may be quite different from the EPS produced by the LAB. Interestingly, EPS biosynthesis by a *B. animalis* subsp. *lactis* strain was promoted in the presence of bile salt, suggesting that induction of EPS biosynthesis by bile salt may be involved in self-protection against this toxic compound (260). While this may be one function of EPS production by bifidobacteria, another appears to be as a fermentable carbohydrate source for other gut bacteria, including bifidobacteria (264). While some have speculated that bifidobacterial EPS may have immunomodulatory effects on the host, it was not found to stimulate lymphocyte proliferation or cytokine secretion, unlike cell wall extracts (8).

While there is no information on EPS production by the nine bifidobacteria whose genomes have been sequenced, they all contain clusters carrying genes predicted to be involved in

EPS biosynthesis. Interestingly, these clusters also contain genes whose annotations suggest involvement in EPS production but which are not present in the EPS gene clusters of the LAB (Fig. 6). This may support a difference in the general EPS structures of bifidobacteria and LAB. The four strains of *B. animalis* subsp. *lactis* and *B. dentium* Bd1 carry EPS gene clusters containing most of the essential genes for EPS biosynthesis, in contrast to the other sequenced bifidobacteria. Compared to the EPS gene cluster of *Lactobacillus rhamnosus* ATCC 9595, these gene clusters contain all of the genes involved, except for glucose transferase (Fig. 6), as well as some extra genes with annotations suggesting that they may have EPS biosynthesis abilities. The other genomes contain EPS gene clusters but are missing more of the genes than *L. rhamnosus* ATCC 9595.

### Lantibiotic Production

Bacteriocin production is an important characteristic for bacterial competition in natural ecosystems. Bacteriocins are small antimicrobial peptides which inhibit the same or closely



TABLE 12. Genes involved in peptidoglycan and rhamnose biosynthesis in bifidobacteria

Functional category and EC no.	KEGG annotation	Locus tag								
		<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bdl	<i>B. animalis</i> subsp. <i>lactis</i>			
		DJO10A	NCC2705				AD011	BI-04 DSM 10140	Bb-12	
Peptidoglycan biosynthesis										
2.5.1.7	<i>murA</i> ; UDP-N-acetylglucosamine 1-carboxyvinyltransferase	BLD_1600	BL1267	Blon_2321	BAD_0183	BDP_0275	BLA_0233	Balac_0245	Balat_0245	Unannotated <sup>c</sup>
1.1.1.158	<i>murB</i> ; UDP-N-acetylmuramate dehydrogenase	BLD_1681	BL1561	Blon_2254	BAD_0294	BDP_0408	BLA_0345	Balac_0364	Balat_0364	BIF_00678
6.3.2.8	<i>murC</i> ; UDP-N-acetylmuramate-alanine ligase	BLD_0182	BL1324	Blon_0857	BAD_1100	BDP_1541	BLA_0775	Balac_1197	Balat_1197	BIF_00646
6.3.2.9	<i>murD</i> ; UDP-N-acetylmuramoylalanine-D-glutamate ligase	BLD_0179	BL1321	Blon_0854	BAD_1103	BDP_1544	BLA_0778	Balac_1200	Balat_1200	BIF_00509
6.3.2.13	<i>murE</i> ; UDP-N-acetylmuramoylalanyl-D-glutamate-2,6-diaminopimelate ligase	BLD_0221	BL1356	Blon_0893	BAD_1085	BDP_1521	BLA_0746	Balac_1168	Balat_1168	BIF_01077
6.3.2.4	<i>dldA</i> ; D-alanine-D-alanine ligase	BLD_1112	BL0345	Blon_0320	BAD_0186	BDP_0283	BLA_0236	Balac_0248	Balat_0248	BIF_01368
6.3.2.10	<i>murF</i> ; UDP-N-acetylmuramoylalanyl-D-glutamyl-2,6-diaminopimelate-D-alanyl-D-alanine ligase	BLD_0177	BL1319	Blon_0852	BAD_1105	BDP_1546	BLA_0780	Balac_1202	Balat_1202	BIF_00378
2.7.8.13	<i>mraY</i> ; phospho-N-acetylmuramoyl-pentapeptide transferase	BLD_0178	BL1320	Blon_0853	BAD_1104	BDP_1545	BLA_0779	Balac_1201	Balat_1201	BIF_00379
2.4.1.227	<i>murG</i> ; UDP-N-acetylglucosamine-N-acetylmuramoyl-(pentapeptide) pyrophosphoryl-undecaprenol N-acetylglucosamine transferase	BLD_0181	BL1323	Blon_0856	BAD_1101	BDP_1542	BLA_0776	Balac_1198	Balat_1198	Unannotated <sup>d</sup>
2.4.1.129	Peptidoglycan glycosyltransferase	BLD_0175	BL1317	Blon_0079, Blon_0850	BAD_0040, BAD_1107	BDP_1548	BLA_0077, BLA_0782	Balac_1204	Balat_1204	BIF_00315
3.6.1.27	<i>bacA</i> ; undecaprenyl-diphosphatase	BLD_0446	BL0721	Blon_1151	BAD_0825	BDP_1149	BLA_1353	Balac_0838	Balat_0838	BIF_01744
3.4.16.4	<i>dacB</i> ; D-alanyl-D-alanine carboxypeptidase	BLD_1845	BL1679	Blon_0546	BAD_0407	BDP_0546	BLA_0471	Balac_0496	Balat_0496	BIF_01386
Rhamnose biosynthesis										
2.7.7.9	<i>ugpA</i> ; UTP-glucose-1-phosphate uridylyltransferase	BLD_0464	BL0739	Blon_1169	BAD_0816	BDP_1163	BLA_1330	Balac_0808	Balat_0808	BIF_00196
2.7.7.24	<i>rfbA</i> ; glucose-1-phosphate thymidyltransferase	BLD_1570	BL0227		BAD_1509	BDP_1860	BLA_0581	Balac_1376	Balat_1376	BIF_01544
4.2.1.46	<i>rfbB</i> ; dTDP-glucose 4,6-dehydratase	BLD_1568	BL0229	Blon_2390	BAD_1507	BDP_1862	BLA_0583	Balac_1378	Balat_1378	BIF_01541
5.1.3.13	<i>rfbC</i> (fused <sup>e</sup> ); dTDP-4-dehydrothiamose 3,5-epimerase	BLD_1569	BL0228		BAD_1508	Unannotated <sup>b</sup>	BLA_0582	Balac_1377	Balat_1377	BIF_01543
1.1.1.133	<i>rfbD</i> (fused <sup>e</sup> ); dTDP-4-dehydrothiamose reductase									

<sup>a</sup> *rfbC* and *rfbD* are fused in bifidobacteria.  
<sup>b</sup> This gene (nucleotides [nt] 2,123,091 to 2,124,545) in *B. dentium* Bdl was not annotated in the original annotation data (CP001750).  
<sup>c</sup> This gene (nt 928,929 to 930,257) in *B. animalis* subsp. *lactis* Bb-12 was not annotated in the original annotation data (CP001853).  
<sup>d</sup> This gene (nt 77,792-78,976, complement) in *B. animalis* subsp. *lactis* Bb-12 was not annotated in the original annotation data (CP001853).

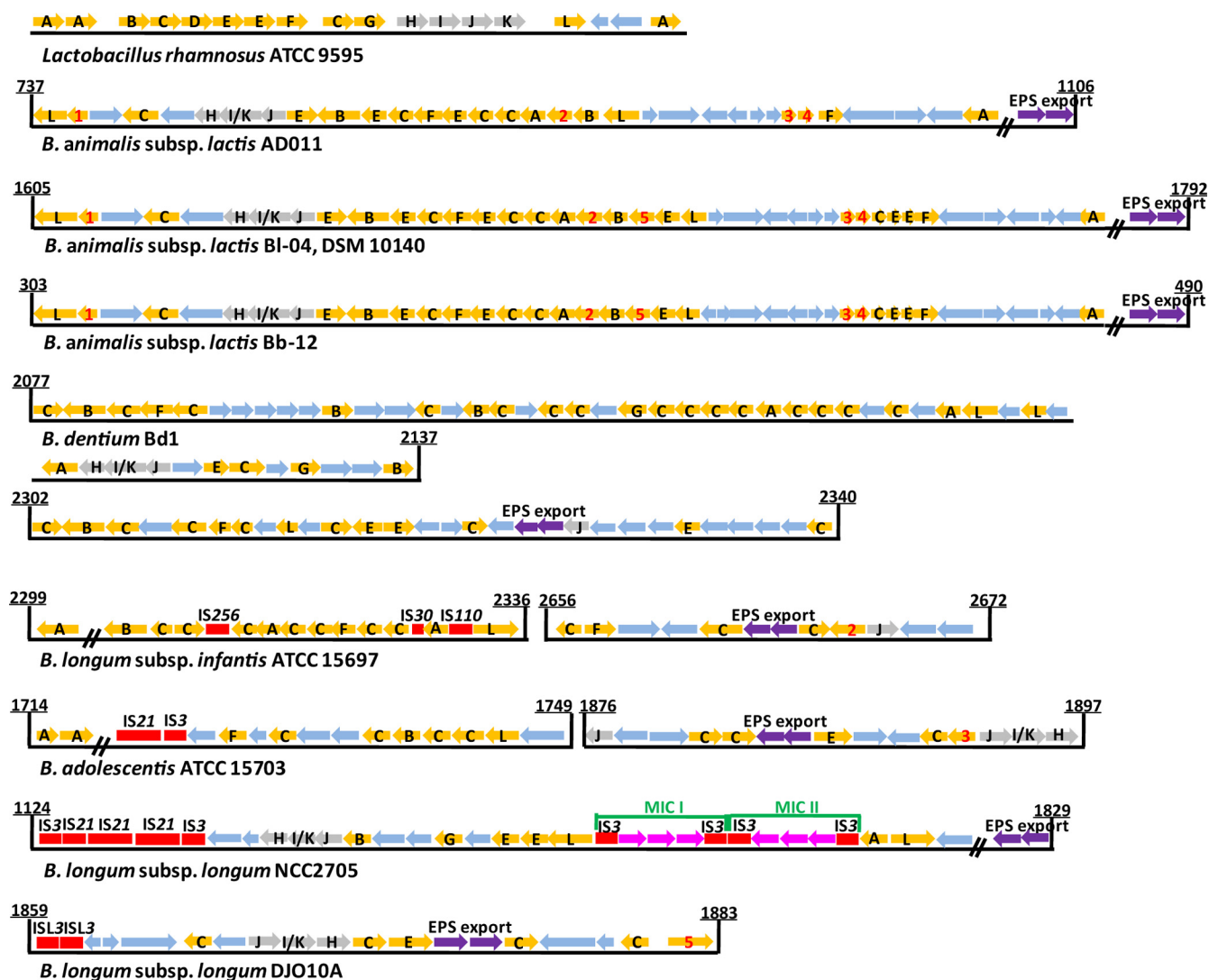


FIG. 6. Comparison of predicted EPS gene clusters in bifidobacterial genomes. The EPS biosynthetic gene cluster in *Lactobacillus rhamnosus* ATCC 9595 was obtained from the work of Peant et al. (228). All genes were categorized according to their potential functions, as follows: A, chain-length determination; B, Wzx flippase for EPS synthesis; C, glycosyltransferase; D, glucose transferase; E, rhamnosyltransferase; F, polysaccharide polymerase/oligosaccharide repeat unit polymerase; G, polysaccharide pyruvyl transferase; H, dTDP-glucose pyrophosphorylase; I, dTDP-4-dehydrorhamnose-3,5-epimerase; J, dTDP-D-glucose-4,6-dehydratase; K, dTDP-4-keto-L-rhamnose reductase; I/K, fused protein of I and K; and L, priming glycosyltransferase/galactosyltransferase/UDP-galactose phosphotransferase. Genes with red numbers are bifidobacterium-specific genes predicted to be involved in EPS biosynthesis, as follows: 1, extracellular exopolysaccharide; 2, UDP-glucose/GDP-mannose dehydrogenase; 3, UDP-N-acetylglucosamine/glucuronate/galacturonate-4-epimerase; 4,  $\beta$ -1,4-galactosyltransferase enhancer; and 5, UDP-galactopyranose mutase. Gray arrows indicate genes involved in dTDP-rhamnose precursor biosynthesis. Sky blue arrows indicate hypothetical genes. The red boxes indicate IS mobile elements. MIC, mobile integrase cassette consisting of three contiguous integrase genes (green) sandwiched by two IS3 mobile elements (161). The genome locations of the gene clusters are indicated in kilobases.

related species (144). Lantibiotics are a very broad-spectrum class of bacteriocins, and their composition differs from that of other bacteriocins, as they contain posttranslationally modified amino acids, such as lanthionine and  $\alpha$ -methyllanthionine, and cyclic structures (44). Recently, the first bifidobacterial lantibiotic was detected and partially purified from *B. longum* subsp. *longum* DJO10A, and it showed a broad antimicrobial spectrum of activity (161). This lantibiotic was encoded by a characteristic lantibiotic operon, with genes encoding production, modification, and regulation functions (161). This operon consists of genes encoding a two-component signal transduction

system (*lanR2* and *lanK*), a lantibiotic prepeptide (*lanA*), a lantibiotic response regulator (*lanR1*), lantibiotic modification enzymes (*lanD* and *lanM*), a lantibiotic immunity protein (*lanI*), and a lantibiotic transporter with predicted protease activity for removal of the leader peptide during secretion (*lanT*) (Fig. 7). The comparative genome analysis of bifidobacteria also showed the homologous remnants of this lantibiotic operon in the genomes of *B. longum* subsp. *infantis* ATCC 15697 and *B. angulatum* DSM 20098 (Fig. 7), suggesting the possibility of widespread lantibiotic production in bifidobacteria.

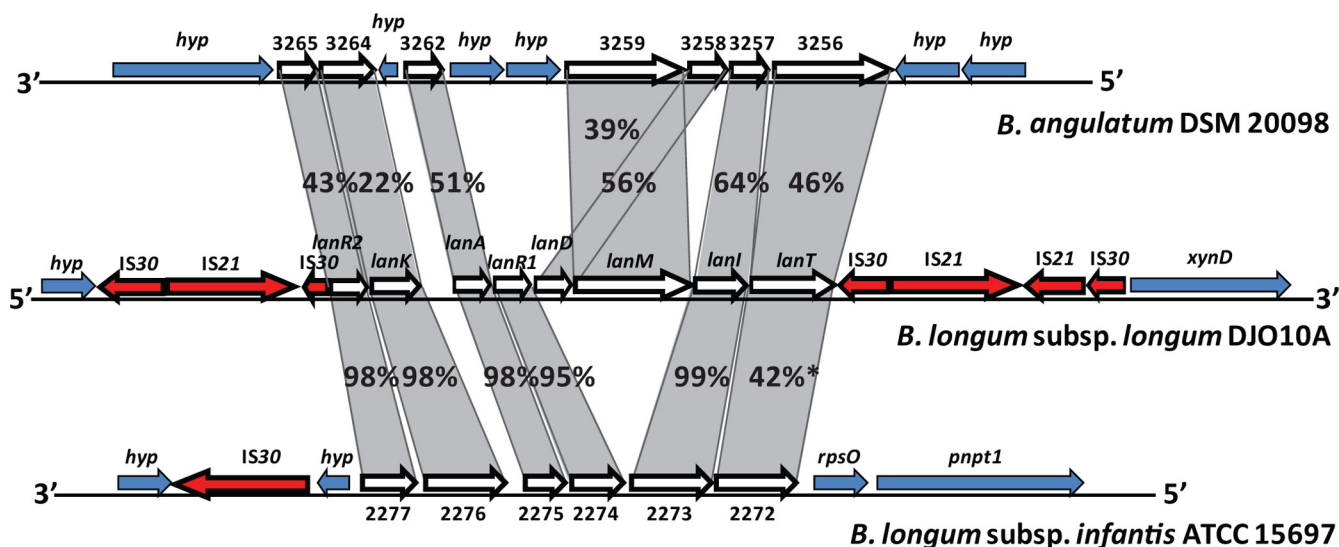


FIG. 7. Comparison of the lantibiotic operon in *B. longum* subsp. *longum* DJO10A with partial operons in *B. longum* subsp. *infantis* ATCC 15697 and *B. angulatum* DSM 20098. Red arrows indicate IS elements, and white arrows indicate the component genes for lantibiotic production, modification, secretion, and immunity. Amino acid sequence identities with *B. longum* subsp. *longum* DJO10A are indicated. The asterisk indicates that the lower overall identity is due to an internal gene deletion, with the common gene regions sharing almost 100% identity. Genes: *lanR2*, response regulator gene; *lanK*, histidine kinase gene; *lanA*, lantibiotic prepeptide gene; *lanR1*, response regulator gene; *lanD*, prepeptide modification enzyme gene; *lanM*, lantibiotic modifying enzyme gene; *lanI*, lantibiotic immunity protein gene; *lanT*, lantibiotic transporter/protease fusion protein gene. The gene numbers of the gene analogs in *B. angulatum* DSM 20098 and *B. longum* subsp. *infantis* are indicated.

### Metabolism of Simple Sugars

The key hexose metabolism pathway in bifidobacteria involves the enzyme fructose-6-phosphate phosphoketolase (F6PPK) and is often called the “bifid shunt,” as bifidobacteria are among the few bacteria to contain this enzyme. Hexoses such as glucose or fructose are metabolized via this pathway to acetate and lactate for energy production (25, 72, 74, 75, 269, 271). The completely sequenced bifidobacterial genomes revealed all the required genes for the F6PPK-mediated bifid shunt, as well as some genes for the trichloroacetic acid (TCA) cycle, except for the genes encoding 2-oxoglutarate dehydrogenase (EC 1.2.4.2), malate dehydrogenase (EC 1.1.1.37), and fumarase (EC 4.2.1.2) (Fig. 4). Since bifidobacteria are among the few intestinal bacteria to have F6PPK, it is the usual diagnostic enzyme used for these bacteria. However, there are some exceptions, such as *Gardnerella vaginalis*, which has a close phylogenetic relationship with the genus *Bifidobacterium* (94), and the Gram-negative bacterium *Acetobacter xylinum* (277). The sequenced bifidobacterial genomes contain genes for the utilization of a wide variety of substrates, such as glucose, fructose, galactose, *N*-acetylglucosamine, *N*-acetylgalactosamine, arabinose, xylose, ribose, sucrose, lactose, cellobiose, melibiose, gentobiose, maltose, isomaltose, raffinose, and mannose (Fig. 4). Carbohydrate fermentation studies with *B. longum* subsp. *longum* NCC2705 and *B. dentium* Bd1 experimentally validated many of these substrates (218, 335). Furthermore, a recent molecular study of ribose utilization by *B. breve* UCC2003 revealed the presence of a functional complete ribose transfer and utilization gene cluster containing *rbsACBDK*, which is regulated by a LacI-type RbsR transcriptional repressor (232).

While the majority of bacteria preferentially utilize simple sugars such as glucose over more complex forms, many bi-

fidobacteria preferentially utilize di- or oligosaccharides (7, 340). The classical substrate comparison is coculture with glucose and lactose, whereby bacteria generally display catabolite repression of lactose utilization until glucose is exhausted. *B. longum* does the opposite, whereby glucose utilization experiences catabolite repression by lactose (143, 219). A microarray analysis of *B. longum* NCC2705 revealed that the only gene downregulated by lactose was a glucose-specific major facilitator superfamily (MFS)-type glucose:H<sup>+</sup> symporter gene (*glcP*), suggesting that only glucose transport experienced catabolite repression by lactose (219). The preference for utilizing di- and oligosaccharides, rather than monosaccharides, is likely an evolutionary adaptation to an environment that is rather devoid of monosaccharides but rich in complex carbohydrates. While lactose is utilized by the host, it is poorly metabolized in many adults and thus frequently reaches the large gut habitat.

Based on complete genome sequence analysis of bifidobacteria and whole-genome microarrays, sugar transportation is predicted to occur via permeases belonging to four families (218). These families are ATP-binding cassette (ABC)-type transport systems for lactose, raffinose, and maltose, a glucose-specific phosphotransferase transport system (PTS) for glucose, MFS systems for lactose, glucose, and sucrose, and the glycoside-pentoside-hexuronide (GPH) cation symporter family for melibiose and pentosides (Fig. 4). Interestingly, the comparative analysis of other complete bifidobacterial genomes also showed that these sugar transport systems are present in all bifidobacterial genomes, except for the four genomes of *B. animalis* subsp. *lactis*, which do not show any gene homologs for PTS-type transport systems (Table 13).

ABC-type sugar transporters are primary active transporters coupling ATP hydrolysis to efficient uptake of sugars across the

TABLE 13. Comparative analysis of COG carbohydrate metabolism category (category G) in bifidobacterial genomes

Functional category and COG no.	No. of genes							COG annotation
	<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	<i>B. animalis</i> subsp. <i>lactis</i>		
	DJO10A	NCC2705				AD011, BI-04, and DSM 10140	Bb-12	
Non-PTS sugar transport system								
COG0395	13	10	16	8	19	5	5	Sugar permeases
COG0477	29	23	41	19	31	15	16	Permeases of the major facilitator superfamily
COG0580	2	2	2	2	2	2	1	Glycerol uptake facilitator and related permeases (major intrinsic protein family)
COG0697	3	3	4	4	4	1	0	Permeases of the drug/metabolite transporter superfamily
COG0738	1	0	1	0	2	0	0	Fucose permease
COG1129	4	4	0	0	0	1	1	ABC-type sugar (aldose) transport system, ATPase component
COG1134	1	1	1	1	0	1	1	ABC-type polysaccharide/polyol phosphate transport system, ATPase component
COG1172	2	2	0	0	2	0	0	Ribose/xylose/arabinose/galactoside ABC-type transport systems, permease components
COG1175	11	8	12	7	16	5	5	ABC-type sugar transport systems, permease components
COG1653	14	10	20	10	0	4	3	Sugar-binding periplasmic proteins/domains
COG1682	1	1	1	1	1	1	1	ABC-type polysaccharide/polyol phosphate export systems, permease components
COG1879	2	2	0	0	2	0	0	Periplasmic sugar-binding proteins
COG2182	1	0	0	0	0	1	1	Maltose-binding periplasmic proteins/domains
COG2211	1	2	4	2	0	1	2	Na <sup>+</sup> /melibiose symporter and related transporters
COG2610	0	0	0	1	4	0	0	H <sup>+</sup> /gluconate symporter and related permeases
COG2814	2	2	2	1	0	1	1	Arabinose efflux permease
COG3833	0	0	0	0	0	1	1	ABC-type maltose transport systems, permease components
COG3839	1	1	2	1	0	1	1	ABC-type sugar transport systems, ATPase components
COG4209	0	0	3	1	3	0	0	ABC-type polysaccharide transport systems, permease components
COG4213	1	2	1	1	3	1	1	ABC-type xylose transport systems, periplasmic components
COG4214	1	2	0	0	2	0	0	ABC-type xylose transport systems, permease components
COG4975	0	0	0	0	0	2	1	Putative glucose uptake permease
Subtotal	93	78	114	61	91	43	41	
PTS sugar transport system								
COG1080	1	1	1	1	1	0	0	Phosphoenolpyruvate protein kinase (PTS system EI component in bacteria)
COG2190	0	0	1	0	1	0	0	Phosphotransferase system IIA components
COG1263	1	1	1	1	1	0	0	Phosphotransferase system IIC components, <i>N</i> - acetylglucosamine specific
COG1925	1	1	1	0	1	0	0	Phosphotransferase system, HPr-related proteins
Subtotal	3	3	4	2	4	0	0	

cell membrane (64, 134). While ABC transporters are the most popular transport system for sugars in bifidobacteria, there is little functional knowledge of these transporters which is specific to these bacteria. A proteomic analysis of *B. longum* showed that a fructose-specific ABC sugar-binding protein (BL0033) and an ATPase (BL0034) were highly produced in the presence of fructose compared to those in the presence of glucose, suggesting that this ABC-type transporter is indeed fructose specific and may play an important role in the uptake of fructose (353). In addition to sugar uptake, ABC-type transporters can also function in efflux of cytotoxic compounds out of the cell. A functional study of two genes in *B. breve* annotated as encoding ABC-type multidrug efflux transporters re-

vealed that coexpression of both genes in *Lactococcus lactis* resulted in enhanced antibiotic resistance activity, suggesting that this efflux pump is functional for antibiotic resistance in bifidobacteria (171). The existence of these multidrug transporters in gut bacteria is likely an evolutionary response to the use of antimicrobials in medicine and possibly to their use in food production and preservation systems.

While ABC-type transporters are present in all living organisms, the PEP-PTS has been detected only in bacteria (134). The first glucose-specific PTS in *B. breve* (66) and a potassium-dependent glucose-specific PTS in *B. bifidum* (152) were demonstrated and characterized biochemically without identification of PTS genes. Transcriptional analysis of a *fru* operon



TABLE 14. Predicted genes for complex carbohydrate and polyol utilization in bifidobacterial genomes

Enzyme	Substrate	No. of genes								
		<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i>	<i>B. adolescentis</i>	<i>B. dentium</i>	<i>B. animalis</i> subsp. <i>lactis</i>			
		DJO10A	NCC 2705	ATCC 15697	ATCC 15703	Bd1	AD011	BI-04	DSM 10140	Bb-12
β-Fructofuranosidase/inulinase	Fructofuran	1	1	1	1	1	1	1	1	1
Cyclomaltodextrinase/neopullulanase	Cyclodextrin	1	1	1	0	0	0	0	0	0
Pullulanase/glycogen-debranching enzyme	Pullulan	2	2	2	2	2	2	2	2	3
Galactofuranosyltransferase	Galactofuran	0	1	0	0	0	1	1	1	1
α-L-Arabinofuranosidase	Arabinofuran	6	4	1	2	3	1	1	1	1
Exo-α-L-arabinofuranosidase II	Arabinofuran	2	2	0	2	1	0	0	0	0
α-N-Arabinofuranosidase	Arabinofuran	1	0	0	0	1	0	0	0	0
Arabinogalactan endo-1,4-β-galactosidase	Arabinogalactan	1	1	1	0	0	0	0	0	0
Arabinan endo-1,5-α-L-arabinosidase	Arabinan	2	2	0	0	1	0	0	0	0
Glycanase/glycogenase	Glycan	1	1	1	1	1	1	1	1	1
1,4-α-Glucan branching enzyme	Glucan	1	1	1	1	1	1	1	1	1
4-α-Glucanotransferase	Glucan	2	2	2	2	2	2	2	2	2
Glucan/glycogen phosphorylase	Glucan	1	1	1	1	1	1	1	1	1
β-1,4-Endoglucanase/cellulase	Glucan	0	0	0	0	2	0	0	0	0
β-1,3-Exoglucanase	Glucan	1	1	1	1	2	0	0	0	0
Xylan esterase	Xylan	1	1	1	1	1	1	1	1	1
Endo-1,4-β-xylanase	Xylan	2	1	0	0	4	0	0	0	1
Extracellular exoxylanase	Xylan	1	2	0	0	0	0	0	0	0
β-Glucuronidase	Glycoside	1	0	0	0	0	0	0	0	0
α-1,4-Glucosidase	Glycoside	1	1	1	1	6	1	1	1	1
Oligo-1,6-glucosidase	Glycoside	1	1	1	1	2	1	1	1	1
β-D-glucosidase	Glycoside	1	1	0	1	1	1	1	1	2
Thermostable β-glucosidase B	Glycoside	1	1	1	1	3	1	1	1	1
Endo-β-N-acetylglucosaminidase	Glycoprotein	1	1	0	0	0	0	0	0	0
N-Acyl-D-glucosamine 2-epimerase	Glycoprotein	1	1	1	0	1	1	1	1	1
Mannitol dehydrogenase <sup>a</sup>	Mannitol	1	0	0	1	1	0	0	0	0
Sorbitol dehydrogenase <sup>a</sup>	Sorbitol	1	0	0	1	0	0	0	0	0
β-N-Acetylhexosaminidase <sup>b</sup>	Glycan	2	2	2	1	0	1	1	1	1
Lacto-N-biose phosphorylase <sup>b</sup>	Glycan	1	1	1	0	0	0	0	0	0
α-L-fucosidase <sup>b</sup>	Glycan	0	0	3	0	1	0	0	0	0
Exo-α-sialidase <sup>b</sup>	Glycan	0	0	1	0	0	0	0	0	0
Total		38	33	24	21	38	17	17	17	20

<sup>a</sup> Polyol utilization genes (161).<sup>b</sup> Predicted to be involved in human milk oligosaccharide utilization (280).

encoding a fructose-specific PTS in *B. breve* revealed that it was specifically induced by fructose (181). Subsequent biochemical verification that fructose is phosphorylated at the C-6 position confirmed the functionality of this fructose PTS in *B. breve*. This differs from the case for *B. longum*, which does not carry a *fru* operon and takes up fructose via an ABC transport system, as discussed above. Indeed, *B. longum* encodes only one PTS, which is glucose specific, suggesting a preference for ABC transporters by this species. Transcriptional analysis of this glucose PTS revealed very little expression, suggesting that glucose may be transported preferentially by the permease system (219). This is substantiated by the catabolite repression of only one gene, the *glcP* glucose permease gene, during growth on lactose.

### Complex Carbohydrate and Polyol Utilization

Successful microbes in any habitat, including the large intestine, must evolve efficient mechanisms for utilizing the available nutrients. Most of the easily digestible simple sugars are generally absorbed or metabolized in the upper gut, and the host-indigestible complex carbohydrates (plant-derived dietary fibers, host-derived glycans, oligosaccharides, resistant starch, cellulose, hemicellulose, xylan, arabinofuran, arabinogalactan,

pectins, and gums) and poorly metabolized polyols are utilized by intestinal bacteria in the lower gut (119). While it is generally assumed that competition for these nutrients is a significant competitive factor for microbes in the gut, this has not been demonstrated experimentally. The analysis of the complete genome sequences of bifidobacteria supports this assumption, as they contain numerous gene clusters predicted to be involved in the metabolism of these substrates (146, 161, 275, 280). Previous carbohydrate fermentation studies demonstrated that multiple species of *Bifidobacterium* have the capacity to utilize a variety of polysaccharides and gums, but in a strain-dependent fashion (54, 266). The capability to utilize host-indigestible complex carbohydrates is believed to be an important competitive feature among bacteria in the large intestine (218).

The sequenced bifidobacterial genomes contain genes predicted to encode many complex carbohydrate utilization enzymes, consistent with the expected biology of these bacteria (Table 14). Many of these genes are located in clusters, and their expression is predicted to be regulated by LacI-type repressors. The nine complete bifidobacterial genomes contain 7 to 11 of these predicted gene clusters, consisting of a LacI-type repressor gene, genes for an ABC-type ATP-dependent trans-

TABLE 15. Occurrence of *B. longum* subsp. *longum* DJO10A polyol utilization gene analogs in other bifidobacteria

Gene <sup>a</sup>	Annotation	Locus tag					
		<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	<i>B. breve</i> DSM 20213	<i>B. catenulatum</i> DSM 16992	<i>B. adolescentis</i> L2-32	<i>B. dentium</i> ATCC 27678
BLD_1696	Mannitol dehydrogenase	BAD_0313	BDP_0423	BIFBRE_00261			BIFDEN_00355
BLD_1697	Mannitol transporter	BAD_0316	BDP_0424	BIFBRE_00262	BIFCAT_01546	BIFADO_00565	BIFDEN_00354
BLD_1698	Lactoylglutathione lyase	BAD_0314		BIFBRE_00263	BIFCAT_01548	BIFADO_00562	
BLD_1699	Sorbitol transporter	BAD_0316	BDP_0424	BIFBRE_00266	BIFCAT_01546	BIFADO_00565	BIFDEN_00354
BLD_1700	Sorbitol dehydrogenase	BAD_0317		BIFBRE_00267	BIFCAT_01545	BIFADO_00566	
BLD_1701	Transcriptional regulator	BAD_0318	BDP_0425	BIFBRE_00268	BIFCAT_01544	BIFADO_00567	

<sup>a</sup> Polyol utilization genes in *B. longum* subsp. *longum* DJO10A.

port system, and various types of catabolic genes (Table 7). Interestingly, the *B. longum* subsp. *longum* genomes contain a large number of genes predicted to be involved in metabolism of plant-derived dietary fibers, such as xylan, arabinan, and arabinofuran, while the other seven genomes contain very few (Table 14). This suggests that *B. longum* subsp. *longum* may be very well adapted to competition in guts with diets rich in plant-derived complex carbohydrates. Conversely, *B. longum* subsp. *infantis* carries the most genes predicted to be involved in utilization of HMOs, consistent with it being a dominant member of a breast-fed infant's intestine.

While polyol utilization is predicted to be an important carbon source for microbes in the large intestine, the genomes of *B. longum* subsp. *longum* DJO10A, *B. adolescentis* ATCC 15703, and *B. dentium* Bd1 are the only ones containing genes predicted to be involved in polyol metabolism (161, 335). The presence of these genes predicts that these bacteria can metabolize mannitol or sorbitol, and microarray analysis and *in vitro* fermentation studies with *B. longum* subsp. *longum* DJO10A and *B. dentium* Bd1 have demonstrated this phenotype experimentally for these strains (335; J. H. Lee and D. J. O'Sullivan, unpublished data). In addition, an analysis of the draft genome sequences of *B. breve* DSM 20213, *B. catenulatum* DSM 16992, *B. adolescentis* L2-32, and *B. dentium* ATCC 27678 revealed analogs for the polyol utilization gene cluster, suggesting its prevalence among different species of *Bifidobacterium* (Table 15).

### Secretion Systems and Cell Surface Proteins

Extracellular and cell surface components are predicted to be important for attachment of bacteria to cells and surfaces. The SignalP program (20, 205) detected signal peptides encoded in more than 200 predicted ORFs, including those for 59 surface-associated lipoproteins and 26 solute-binding proteins of ABC transport systems, in *B. longum* subsp. *longum* NCC2705 (275). Interestingly, these predicted secretion proteins are analogous to several polymer hydrolysis enzymes, such as xylanase, arabinosidase, arabinogalactan endo-1,4- $\beta$ -galactosidase, and *N*-acetylglucosaminidase, which could function to digest plant cell wall material and extracellular polysaccharides to smaller sugars and oligosaccharides.

A functional analysis of all nine bifidobacterial genomes by use of the updated KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway database revealed that the protein export systems of bifidobacteria contain Sec-dependent pathways, type I and II signal peptidases, and components of signal recognition particle (SRP)-dependent pathways. These pathways

function together for protein secretion, whereby the SRP receptor is proposed to bind to the signal peptide of the prepeptide to facilitate the action of the signal peptidases and the Sec translocase, located in the cell membrane, for secretion of the cleaved peptide (61).

Many surface proteins are covalently linked to the peptidoglycan layer through the action of sortase transpeptidases. The sortase enzymes recognize a conserved LPXTG motif in proteins targeted for cell wall anchoring, cleave proteins between the threonine-glycine bonds, and catalyze transpeptidation to a peptidoglycan cross-linking spacer (146). The LPXTG motif is a signature of one class of cell surface anchoring proteins (317). While the majority of the LPXTG-type proteins encoded by the bifidobacterial genomes are unknown, one is a weakly conserved homolog of FimA and is encoded in all of the genomes except for that of *B. longum* subsp. *infantis*. The LPXTG-type proteins are potential cell surface proteins that may be involved in attachment to cells or mucus in the gut. Interestingly, while the bifidobacterial genomes share some analogs encoding putative cell surface proteins with LPXTG motifs, there are also species-specific genes encoding LPXTG-type proteins (Table 16). While both strains of *B. longum* subsp. *longum* carry the same gene analogs for LPXTG-type proteins, one of them, in strain NCC2705, is missing the 3' area of the gene that encodes the LPXTG motif. Given that strain DJO10A was sequenced before extensive culturing outside the gut, unlike strain NCC2705, this observation supports the tendency of bacteria to undergo genome reduction during pure culture growth in regions that are no longer needed for the new environment (161).

Most *Eubacteria* and *Archaea* have a *tad* (tight adherence) locus involved in pilus construction and believed to be important for adherence to cell surfaces or mucus (135). Interestingly, all of the bifidobacterial genomes contain a few highly conserved sortase genes and gene analogs of *tadABC*, arranged in an operon, as well as *tadV*, which may be involved in pilus assembly for cell surface adherence. The *tadA* gene analog encodes a putative ATPase that is proposed to be involved in the secretion of a FimA prepilin, which is also predicted to be present in bifidobacteria. The *tadBC* genes are proposed to encode a transport system for the prepilin, and *tadV* encodes a putative peptidase for maturation of the prepilin. This *tad* locus is similar in structure to other loci in other actinobacteria, such as *Streptomyces coelicolor* and *Corynebacterium diphtheriae* (135). These surface protein export

TABLE 16. Genes predicted to be involved in secretion and cell attachment in bifidobacteria

Functional category and gene annotation <sup>a</sup>	Locus tag <sup>b</sup>								
	<i>B. longum</i> subsp. <i>longum</i>		<i>B. longum</i> subsp. <i>infantis</i> ATCC 15697	<i>B. adolescentis</i> ATCC 15703	<i>B. dentium</i> Bd1	<i>B. animalis</i> subsp. <i>lactis</i>			
	DJO10A	NCC2705				AD011	BI-04	DSM 10140	Bb-12
Sortases									
<i>srtA1</i>	BLD_0779 BLD_1287	BL1028 BL0498	Blon_1841 Blon_0162	BAD_0087 BAD_1470	BDP_0867 BDP_0197 BDP_2002 BDP_0536 BDP_0142 BDP_0036	BLA_1023 BLA_0681	Balac_1023 Balac_1486	Balat_1023 Balat_1486	BIF_01167 BIF_01042
<i>srtA2</i>	BLD_1385	BL0591	Blon_0075 Blon_1280	BAD_0036		BLA_0073	Balac_0082	Balat_0082	BIF_01566
<i>srtA3</i>	BLD_1458								
<i>srtA4</i>	BLD_1469	BL0676		BAD_0122 BAD_1551	BDP_2188 BDP_0278	BLA_0554	Balac_1349	Balat_1349	BIF_00667
LPXTG-type cell surface-anchoring secretion proteins	BLD_1189 BLD_1468 BLD_1637 BLD_1638 <sup>c</sup> BLD_1511 <sup>c</sup>	BL0387 BL0675 BL1539 BL1540 <sup>c</sup> BL0146 <sup>c,d</sup>	Blon_0283     Blon_1005 <sup>c</sup> Blon_1227 <sup>c</sup> Blon_2163 <sup>c</sup>	BAD_1467     BAD_1451 <sup>c</sup>	      BDP_0144 <sup>c</sup> BDP_0200 <sup>c</sup> BDP_0276 <sup>c</sup> BDP_0277 <sup>c</sup> BDP_0535 <sup>c</sup>	BLA_0679 BLA_0680	Balac_1484 Balac_1485 <sup>f</sup>	Balat_1484 Balat_1485 <sup>f</sup>	BIF_01518 <sup>c</sup> BIF_00999 BIF_00998 <sup>f</sup>
						BLA_1540 <sup>c</sup>	Balac_1618 <sup>c,f</sup>	Balat_1618 <sup>c,f</sup>	BIF_01674 <sup>c,f</sup>

<sup>a</sup> Based on the *B. longum* subsp. *longum* DJO10A annotation.  
<sup>b</sup> Genes on the same line are analogs.  
<sup>c</sup> Species-specific predicted LPXTG-containing cell surface protein.  
<sup>d</sup> LPXTG motif missing because of a predicted ISL3 deletion event.  
<sup>e</sup> LPXTG motif is missing.  
<sup>f</sup> No predicted secretion ability.

systems and pilus construction capabilities of bifidobacteria may play an important role in their attachment to mucus or cells in the intestine.

Stress Response

**Oxygen tolerance.** Even though bifidobacteria are generally described as strict anaerobes, some of them can tolerate some oxygen (269, 293). Their oxygen sensitivities are variable (73, 137, 284), with the fermented milk-adapted organism *B. animalis* subsp. *lactis* having the highest oxygen tolerance (185). While anaerobic bacteria differ in their sensitivity to oxygen, some have different degrees of tolerance due to the activities of some enzymes to remove reactive oxygen species (ROS), such as NADH oxidase, NADH peroxidase, and superoxide dismutase (SOD). SOD can eliminate superoxide radicals, and previous studies have reported SOD activity in bifidobacteria (284, 285, 306). However, the nine completed genome sequences of bifidobacteria do not reveal any SOD-related genes. The discrepancy may be explained by the proposal that SOD activity measurements in bifidobacteria were probably false-positive results, based on comparisons with known SOD enzymes (42, 160). This proposal is supported by the genome analysis.

Although previous studies have shown that bifidobacteria utilize NADH oxidase/peroxidase-like systems for the protection of their cells from ROS (284), the complete genome sequences of bifidobacteria revealed a gene analog only for the NADH oxidase gene. The reaction of oxygen and NADH with NADH oxidase produces and results in accumulation of hy-

drogen peroxide (H<sub>2</sub>O<sub>2</sub>), which has many destructive properties, including inhibition of the F6PPK enzyme (73). The absence of catalase activity (or gene analogs for this enzyme) in bifidobacteria indicates that some other mechanism must be present to prevent the accumulation of H<sub>2</sub>O<sub>2</sub> during exposure to oxygen. Previous studies indicated that different *Bifidobacterium* species did not accumulate H<sub>2</sub>O<sub>2</sub> during exposure to oxygen (284, 285, 306). These studies proposed that this was due to NADH peroxidase activity, which converts H<sub>2</sub>O<sub>2</sub> to water by utilizing NADH. However, none of the genome sequences of bifidobacteria contain gene analogs for NADH peroxidase, suggesting that there may be an alternative mechanism. Interestingly, all of the genome sequences of bifidobacteria, except for that of *B. adolescentis* ATCC 15703, reveal peroxiredoxin family alkyl hydroperoxide reductase gene analogs, which have been shown to be involved in the reduction of H<sub>2</sub>O<sub>2</sub> in *E. coli* and *Streptococcus mutans* (233, 278). An alternative system for H<sub>2</sub>O<sub>2</sub> reduction to H<sub>2</sub>O is the reduction of thioredoxin by thioredoxin reductase and NADH. Thioredoxin systems are prevalent in nature and are proposed to have multiple functions, including protection from oxidative stress (10, 198). All of the genomes of bifidobacteria carry gene analogs for thioredoxin and thioredoxin reductase, supporting this alternative mechanism for removal of H<sub>2</sub>O<sub>2</sub> from bifidobacteria during exposure to oxygen, and this could explain the limited tolerance of many bifidobacteria to air. In addition, the bifidobacterial genomes contain oxidative damage repair-related genes, including those for nucleoside triphosphate pyrophosphohydrolase (MutT) and DNA-binding ferritin-like

protein (Dps), which are involved in removing hydroxyl radicals (91).

**Acid tolerance.** The  $F_0F_1$ -type ATPase system is involved in acid tolerance by pumping protons out of the cell. All nine bifidobacterial genomes contain a putative *atp* operon which carries all genes needed for a complete  $F_0F_1$ -type ATPase system. Previous studies have also demonstrated the activity of the ATPase system in different species of *Bifidobacterium* in response to acid stress (175, 326). While all nine bifidobacterial genomes contain gene analogs for this system, the acid tolerance of *B. animalis* subsp. *lactis* strains is superior to that of all other members that colonize the gut. Interestingly, the genomes of the gut bifidobacteria do not reveal additional stress-related genes, suggesting that these bacteria may have an alternative expression pattern for the *atp* operon, such that it is active before exposure to more acidic conditions. Alternatively, other novel mechanisms may be present in these “fermentation-friendly” bifidobacteria. Recent genome analysis of *B. dentium* Bd1 revealed another acid tolerance system, the glutamate-dependent acid resistance system 2, which encodes a glutamate decarboxylase (GadB) and a glutamate/gamma-aminobutyrate antiporter (GadC) (335). This likely gives this oral resident more tolerance to acid than that of the gut bifidobacteria and possibly aids in its ability to induce acid damage to tooth enamel in dental caries.

**Temperature tolerance.** While bifidobacteria do not encounter temperature stresses in their normal intestinal environment, they are subjected to temperature stresses when utilized for probiotic applications. All nine complete genome sequences of bifidobacteria carry several temperature stress-related gene analogs, including genes encoding the chaperone families Hsp100 (ClpBCX), Hsp70 (DnaK, GrpE, and DnaJ), and Hsp60 (GroEL/GroES complex) and the SOS response (LexA, RuvA, RecA, and MutY).

The transcription of all genes involved in the Hsp60 and Hsp70 chaperone families of *Firmicutes* and *Proteobacteria* is regulated by the HrcA-CIRCE (controlling inverted repeat of chaperone expression) system (328). The *Actinobacteria*, including all of the sequenced bifidobacteria, utilize the HrcA-CIRCE system for the transcriptional regulation of only some of the Hsp60 chaperone genes (*groEL*, *groES*, and *dnaJ2*). All of the genomes contain a gene encoding a putative HrcA protein, which is a transcriptional repressor of heat shock chaperone genes. The binding site for HrcA is a characteristically conserved inverted repeat (CIRCE) with the consensus sequence TTAGCACTC-N<sub>9</sub>-GAGTGCTAA (16, 356). CIRCE-like motifs were found in the promoter regions of *dnaJ2* (ATTAGCACTC-N<sub>9</sub>-GAGTGCTAAT) and *groES* (GTTAGCACTC-N<sub>9</sub>-GAGTGCTAAC) in bifidobacteria. In addition, a recent computational analysis of the unpublished *B. breve* UCC2003 genome revealed the presence of conserved CIRCE-like motifs in the promoter regions upstream of the *hrcA* and *groEL* genes (355). Interestingly, two CIRCE-like motifs were detected in the promoter region of the *groEL* gene, and one CIRCE-like motif, as well as a ClgR-binding site, was found in the promoter region of the *hrcA* gene. The presence of a ClgR-binding site suggested that expression of the *hrcA* gene may be controlled by the ClgR transcriptional activator. Interestingly, the *groEL* genes are clustered with *cspA* (cold shock protein gene) in bifidobacteria, which is unusual but has also been reported

for *Leifsonia xyli* subsp. *xyli* CTCB07 (192) and *Streptomyces coelicolor* A3(2) (22). While CIRCE-like elements were found in the promoter regions of *dnaJ2* (ATTAGCACTC-N<sub>9</sub>-GAGTGCTAAT) and *groES* (GTTAGCACTC-N<sub>9</sub>-GAGTGCTAAC) in bifidobacteria, the promoter region of the *cspA*-*groEL* gene cluster contains two different inverted repeats. These are 10-bp (GCCACCATCA) and 8-bp (CGTTCCT) inverted repeats, with one 5-bp direct repeat (CGCGA), all of which were previously described for *B. breve*, which was also shown to cotranscribe *cspA* and *groEL* (323, 329). A recent analysis of *groEL* indicated that it has very different expression levels from those of *cspA* and also identified an HrcA-regulated promoter (355). *In vitro* binding of purified HrcA with promoter regions of *hrcA*, *groEL*, and *groES* substantiated this observation. Analysis of *B. breve* gene expression under different stress conditions (42 to 50°C, ethanol stress, and osmotic stress) by use of a whole-genome microarray showed that the expression of the *cspA* gene was significantly expressed only during exposure to 50°C, while expression of the *groEL* gene was high under all stress conditions, supporting the presence of its own promoter (355).

The Hsp70 chaperone family also utilizes another characterized transcription regulatory mechanism, designated HAIR (HspR-associated inverted repeat), with the consensus sequence CTTGAGT-N<sub>7</sub>-ACTCAAG (37, 101). In *Actinobacteria*, HspR is an autoregulatory repressor of the *dnaK* operon (encoding the Hsp70 chaperone), which contains the *hspR* gene and the *clpB* gene. Recently, HAIR-like motifs were found in the promoter regions of the *dnaK*, *clpB*, and *clgR* genes of *B. breve* and were demonstrated experimentally to bind HspR, substantiating the involvement of this regulatory mechanism (355). Interestingly, the promoter regions of the *nfo* and *hrdB* genes also contained HAIR-like motifs but did not bind HspR. Since the HAIR-like motifs in the promoter regions of *dnaK*, *clpB*, and *clgR* have 3'-end (T-rich) and 5'-end (A-rich) extensions, unlike the HAIR-like motifs in *nfo* and *hrdB*, a role for these extensions in HspR binding was suggested, which was subsequently substantiated by a mutation analysis (355). This proposed a new consensus sequence for HAIR motifs in bifidobacteria, to include these extensions (AAASTTGAGYSW-N<sub>5</sub>-CTCAASTTTT). While the three HspR-repressed stress genes were found to be upregulated by mild heat stresses, they were significantly upregulated during very high heat stresses (50°C). They were also shown to be induced by ethanol and osmotic stresses (355). Recently, heat shock-tolerant mutants of *B. longum* subsp. *longum* were obtained by deliberate exposure to heat stress, and a transcriptome analysis revealed overexpression of the *dnaK* operon and *clpB*. Further analysis showed that the HspR repressor contained point mutations which were proposed to prevent its repressor activity, a phenotype that could be reversed by complementation with a wild-type *hspR* gene (23).

Interestingly, the bifidobacterial genomes carry two different *dnaJ* gene analogs (*dnaJ1* within the *dnaK* operon and *dnaJ2* within the GroEL/GroES gene cluster). The presence of two *dnaJ* genes, at the same locations, has also been found for the other sequenced genomes from the *Actinobacteria* phylum (323). However, the other major bacterial phyla within the GI tract, *Firmicutes* and *Proteobacteria*, carry only a single *dnaJ* gene, within the *dnaK* operon. Multiple *dnaJ* genes can also be



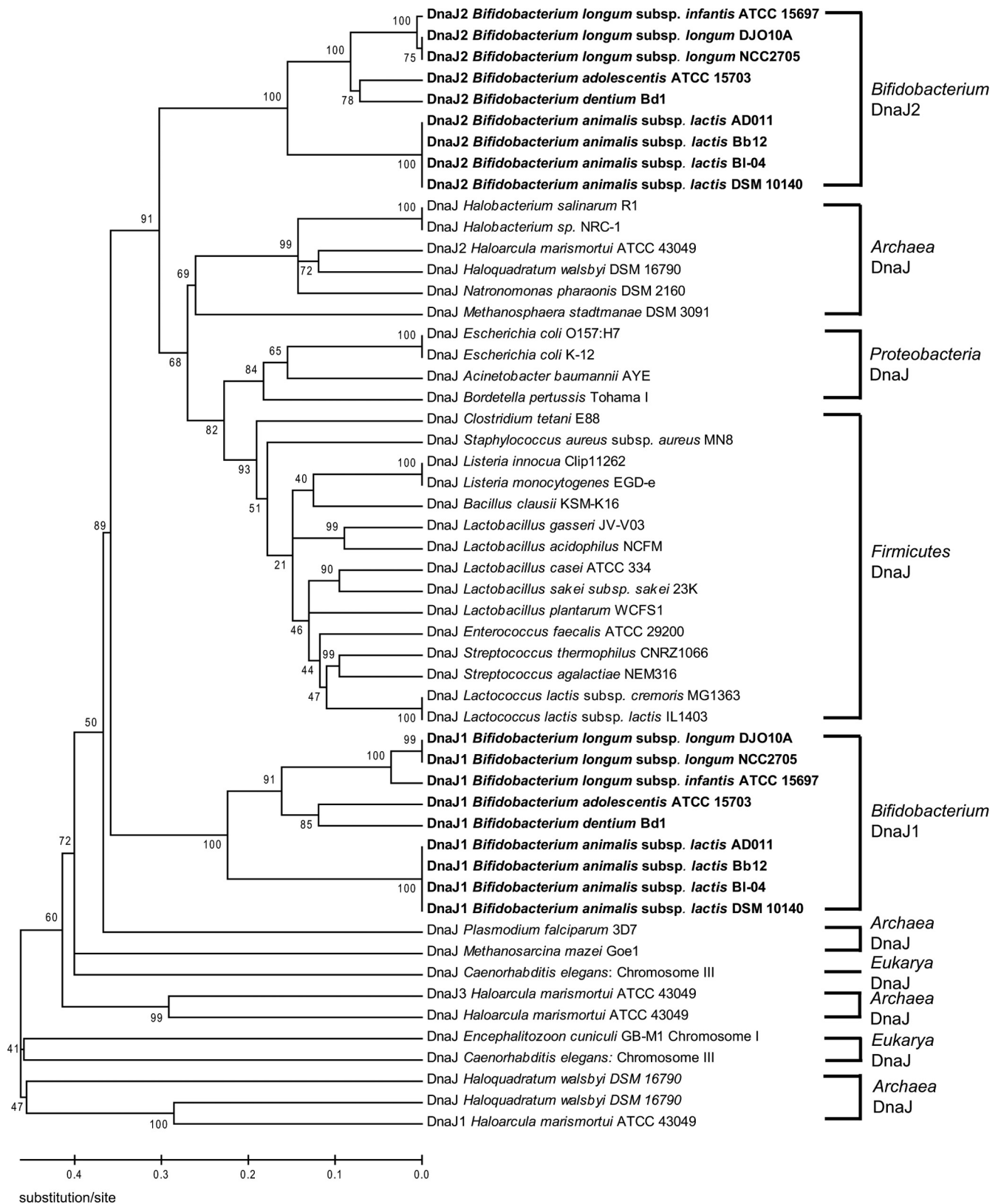


FIG. 8. Phylogenetic analysis of DnaJ proteins in *Proteobacteria*, *Firmicutes*, *Archaea*, and *Eukarya* compared with the two DnaJ proteins encoded by the nine complete bifidobacterial genomes (in bold).

found in the sequenced *Archaea* genomes and in the *Eukarya*. A phylogenetic tree of DnaJ protein sequences shows that bifidobacterial DnaJ1 clusters more closely with DnaJ proteins from *Archaea* and *Eukarya*, whereas DnaJ2 is not related to these, clustering on a similar branch with *Proteobacteria*, *Firmicutes*, and other *Archaea* DnaJ proteins (Fig. 8). This suggests that the bifidobacterial *dnaJ* genes have different ancestral origins.

A number of gene analogs for the Hsp100 chaperone system are present in the nine bifidobacterial genomes. These include *clpC*, which is clustered with a *uspA* analog (encoding universal stress protein), a *clpP1-clpP2* operon, and *clpX*. The expression of this gene system has previously been shown to be regulated at the transcriptional level in bacteria by the ClgR transcription activator, which binds a palindromic sequence motif (CGC-N<sub>5</sub>-GCG; a ClgR-binding motif) in the promoter regions (84, 337). A *clgR* gene is also present in the bifidobacterial genomes, as well as a ClgR-binding motif upstream of the *clpP1* gene in the apparent *clpX-clpP1-clpP2* operon, which has previously been shown to be regulated by ClgR in bifidobacteria (337). Interestingly, this ClgR-binding motif upstream of *clpC* contains one mismatch (CTC-N<sub>5</sub>-GCG), which may explain why extracts of *clgR* overexpressed in *E. coli* did not bind this promoter region alone but did so when combined with extracts of *B. breve*, suggesting the requirement for a cofactor or helper protein for ClgR binding (331). Recently, an N-terminally truncated ClgR protein was shown to form complexes without additional cofactors with the upstream regions of the *clpC* and *clpP1* genes, suggesting possible folding differences of this protein in *E. coli* and confirming that ClgR binds and regulates these promoters (355). A subsequent mutation analysis of the proposed ClgR-binding motif in the promoter region of *clpC* demonstrated that specific bases are important for interaction with ClgR, suggesting an updated consensus binding motif for bifidobacteria, i.e., TNCGCTNNNGGCG NAA, which is larger than the motif in *Streptomyces* but similar to the proposed motif in *Corynebacterium* (355).

Heat stresses are known to induce genes involved in the SOS response (85). For *B. breve*, a transcriptome analysis indicated that a number of SOS genes were upregulated by heat stress, including *recA* and *recX*, which are located directly downstream from the *clgR* two-gene cluster (355). This suggested the possibility that induction of *clgR* during heat stress may carry through to the downstream genes, which was substantiated by the similar induction levels for the four genes. This gene organization, *clgR-hyp-recA-recX*, can be seen in all the bifidobacterial genomes. A LexA SOS repressor is encoded by all the bifidobacterial genomes, and it has been shown to be induced by heat stress in *B. breve* (355). While a potential LexA-binding motif is present upstream of *recA*, suggesting a regulatory role, this has not been investigated experimentally.

Small heat shock proteins (sHSPs) are important to prevent irreversible protein denaturation caused by cellular stresses (203). Members of this protein family usually range from 12 to 30 kDa, but larger members have been found. They are not highly conserved, except for conserved  $\alpha$ -crystalline domains. While most bacteria encode at least one sHSP, some, such as *Helicobacter pylori*, do not (203). Computational genome analysis of bifidobacterial genomes revealed that *hsp20* gene analogs encoding a family of sHSPs are present in four of the nine

completed genomes (all *B. longum* subspecies and *B. adolescentis*), and a BLAST analysis also showed analogs in *B. breve*, *B. bifidum*, and *B. angulatum*. In addition, hybridization analysis revealed putative *hsp20* analogs in *B. catenulatum* and *B. longum* subsp. *suis* (327). This suggests that *hsp20* gene analogs are not widely distributed in bifidobacteria but are relatively conserved in the genus. A phylogenetic analysis of Hsp20 protein sequences in bifidobacteria revealed that they grouped with proteins from the *Firmicutes* rather than the *Actinobacteria*, suggesting that these *hsp20* gene analogs may have been obtained via horizontal gene transfer from *Firmicutes*. Expression analysis of the *hsp20* analog in *B. breve* showed significant induction during heat and osmotic stresses (327, 355). However, sequence analysis of its upstream region did not reveal promoter-like sequences or other known motifs, suggesting a novel regulatory mechanism.

## GENOME ADAPTATION OF BIFIDOBACTERIA TO THEIR HABITAT

### Bifidobacterial Genomes Reveal Features Consistent with a GI Tract Habitat

The microbial population of the human large intestine is limited primarily to host-indigestible nutrients, such as HMOs in infants and plant-derived complex carbohydrates and polyols in adults. In this habitat, intestinal bifidobacteria are efficient utilizers of these types of nutrients, and a significant part of their genomes is dedicated to these capabilities, indicating their adaptation to this habitat (161, 275, 280).

Mammals, including humans, rely primarily on milk for dietary purposes during the infant stage of life. Fecal culturing studies of breast-fed human infants typically show a flora high in bifidobacteria (21, 299). However, the flora is very dynamic during the first year of life, as demonstrated using 16S rRNA gene sequence and PCR-denaturing gradient gel electrophoresis analyses (87). While a nonculturing study of two infants found that both contained high levels of bifidobacteria for up to 6 months during their breast-feeding period, these infants showed variable levels after that. The prominent levels of bifidobacteria in infants were substantiated by analyzing the feces of 1,032 1-month-old breast-feeding infants by quantitative reverse transcription-PCR and finding levels ranging from 10<sup>6</sup> to 10<sup>11</sup> CFU/g feces in 98.6% of subjects (229). Interestingly, a recent study of 14 infants over the first year of life found a low abundance of *Actinobacteria* (1.28%) by sequence analysis of cloned 16S rRNA genes (217). In addition, this study developed a microarray with the cloned fragments and used it to follow the microbial profile for 1 year, and it did not find very high levels of bifidobacteria. This may reflect low bifidobacterial levels in this small group of infants or possibly some biases in the fecal DNA preparation whereby *Actinobacteria* were not represented quantitatively. It should be noted that five of these infants were delivered by Caesarian section rather than vaginally, and these infants had the lowest levels of bifidobacteria. A metagenomic study that included four Japanese infants of less than 7 months of age found that two of them contained a very large bifidobacterial population, one had a smaller but still significant population, and one did not show any bifidobacteria (156). This again demonstrates the variability of bifidobacterial

populations in infants. In many infants, *B. longum* subsp. *infantis* is a very common inhabitant, while *B. longum* subsp. *longum* largely replaces this subspecies in adults (174, 247). A comparative genome analysis of both of these subspecies showed significantly more genes predicted to be involved in HMO utilization in *B. longum* subsp. *infantis* than in *B. longum* subsp. *longum* and, conversely, significantly more genes involved in utilization of plant-derived complex carbohydrates and polyols in *B. longum* subsp. *longum* (Table 14). Specifically, *B. longum* subsp. *infantis* contains seven genes predicted to be involved in HMO utilization, compared to three genes in *B. longum* subsp. *longum*. In addition, the intestinal *B. longum* subsp. *longum* strain DJO10A contains nine arabinofuranosidase and three xylanase genes, predicted to be involved in utilization of plant-derived complex carbohydrates, while *B. longum* subsp. *infantis* contains just one arabinofuranosidase gene. Furthermore, *B. longum* subsp. *longum* carries two polyol utilization genes, while *B. longum* subsp. *infantis* does not carry any (Table 14). While ecological conclusions cannot be drawn from only a few genome sequences, these genome characteristics are intriguing, as they suggest that the *B. longum* subsp. *infantis* genome may be well adapted for a breast-feeding infant's gut, while the *B. longum* subsp. *longum* genome encodes metabolic characteristics consistent with an adult gut.

Another genome insight into the adaptation of *B. longum* subsp. *infantis* is that it contains a complete urease gene cluster, which is not present in any of the other bifidobacterial genomes. This was previously demonstrated functionally in a study showing that of more than 100 strains of different species of *Bifidobacterium*, all 9 strains of *B. longum* subsp. *infantis* produced urease and none of the others did, except for 1 strain of *B. bifidum* among 12 strains tested for this species (302). Given that human milk contains a significant portion of its nitrogen in urea (78), this gene cluster should enable *B. longum* subsp. *infantis* to utilize urea for its nitrogen needs. Given the nutritional need of a growing infant for human milk protein, the ability to utilize the available urea may be a competitive advantage.

#### Genome Adaptation of Intestinal Bifidobacteria to Environments Outside the Gut

Of the nine completed genome sequences from bifidobacteria, only one (*B. longum* subsp. *longum* DJO10A) is from an isolate that was purposely minimally cultured outside the gut prior to sequencing (161). It is intriguing that this strain contains the most genes predicted to be involved in the metabolism of complex carbohydrates and polyols (Table 14). Conversely, the complete genome sequences of four strains of *B. animalis* subsp. *lactis* revealed that they have the fewest genes predicted to be involved in the utilization of these substrates. Specifically, these strains have limited or no predicted ability to metabolize polymers consisting of arabinofuran, arabinogalactan, arabinan, cyclodextrin, xylan, or sugar alcohols, and they have the only genomes missing a gene encoding  $\beta$ -1,3-exoglucanase, thereby limiting their ability to metabolize glucan-based polymers (Table 14). While *B. animalis* subsp. *lactis* was isolated from yogurt, it is not normally associated with traditional fermented milks and likely adapted to this environment following the inclusion of *B. animalis* subsp. *animalis* in yogurts

for probiotic purposes. Interestingly, it has a high tolerance to oxygen (185), which is unusual for this genus, with the other exception being *B. psychroaerophilum*, which can even grow on agar media under aerobic conditions (291). While the origin of *B. animalis* subsp. *animalis* is animal intestines, the origin of *B. animalis* subsp. *lactis* is likely the dairy fermentation environment, and it was first isolated from yogurt. The prolonged exposure of this subspecies to dairy fermentation environments is further supported by the reduced size of the genomes of this subspecies, which is a phenomenon that has been found to occur in organisms that are in less-complex environments where fewer features are needed to be successful, such as the genome reduction of *Buchnera aphidicola*, which is predicted to be due to environmental changes (230). This may explain the inability of these bacteria to persist in the intestines of human subjects, as they have been observed to disappear rapidly after stoppage of eating foods containing these bacteria (88, 215, 300, 319).

In addition to the differences in complex carbohydrate and polyol utilization, the genome of *B. longum* subsp. *longum* DJO10A is the only one of the nine to carry a gene cluster involved in bacteriocin production, specifically that of a broad-spectrum lantibiotic, and gene clusters for arsenic resistance (161). These are features that would be beneficial for an intestinal habitat, as the ability to produce a lantibiotic can give the organism a competitive edge over other intestinal bacteria, and arsenic resistance is common in intestinal bacteria due to low levels of arsenic present in human diets. The predicted superior arsenic resistance of *B. longum* subsp. *longum* DJO10A was confirmed experimentally by comparison to strains of *B. animalis* subsp. *lactis* that do not contain arsenic resistance gene clusters (161).

Genome adaptation in bifidobacteria via genome reduction was suggested previously by comparative genome analysis of two strains of *B. longum* subsp. *longum*, namely, NCC2705, from a culture collection, and DJO10A, cultured minimally outside the gut (161). This analysis revealed that strain NCC2705 has a smaller genome, and it was predicted by a base deviation index analysis to have lost several gene clusters that are predicted to be involved in characteristics important for survival and competition in the gut. This was evaluated experimentally by culturing strain DJO10A in the laboratory, and after 1,000 generations, it was found to have lost two large genome regions, including one that encoded the lantibiotic, in a deletion event very similar to the deletion predicted for strain NCC2705. This deletion mutant of strain DJO10A was shown in a simulated fecal growth environment to have lost a significant proportion of its competitive abilities against other intestinal bacteria (161). This demonstration of genome reduction in bifidobacteria highlights the need to minimize pure culture growth of strains if they are to be utilized as probiotics and expected to function optimally in the GI tract following their ingestion. While commercial production requires large-scale culturing, this limitation of bifidobacteria could be minimized by always starting each culture batch from a frozen stock and developing culturing media that are more representative of the environment of the large gut, such as minimizing exposure to oxygen and acids as well as using a varied complex carbohydrate nutrient profile rather than simple sugars.



## CONCLUDING REMARKS

Since bifidobacteria were first discovered in the feces of breast-fed infants more than 100 years ago, there has been a lot of interest in the potential health benefits these bacteria may afford the large intestine. However, surprisingly little is known about which features are important for their survival, colonization, competition, and interactions with other members of the intestinal microbiota. To further this understanding, a genome sequence analysis of bifidobacteria has provided novel molecular insights into their overall characteristics, physiology, and interactions with their habitat. This is exemplified by the presence in bifidobacterial genomes of many genes involved in the uptake and utilization of host-indigestible complex carbohydrates and polyols, supporting their genome adaptation to the human large intestine, where these are the predominant nutrient sources. While the number of genomes is too small to make reliable ecological predictions, the genomes of the two subspecies of *B. longum* do suggest a possible adaptation, as *B. longum* subsp. *infantis* contains a larger number of genes predicted to be involved in human milk oligosaccharide utilization, consistent with it being a dominant member of a breast-fed infant's bifidobacterial flora. Conversely, *B. longum* subsp. *infantis* contains fewer genes predicted to be involved in utilization of plant-derived complex carbohydrates and polyols, consistent with *B. longum* subsp. *longum* being a prominent member of an adult's bifidobacterial flora. However, since the culturing history of the *B. longum* subsp. *infantis* strain prior to sequencing is not known, it is possible that the absence of some of these genes may be due to attenuation. The only bifidobacterial genome sequence that was obtained from a strain that was minimally cultured outside the gut was from *B. longum* subsp. *longum* DJO10A, and it carried significantly more genes predicted to be involved in the utilization of complex carbohydrates and polyols, resistance to arsenic, and bacteriocin production, all of which are features predicted to be beneficial for competition in the human GI tract. Growth of this strain in the laboratory demonstrated experimentally that it can lose these features through genome deletion events. This may explain the small genome size of *B. animalis* subsp. *lactis*, which contains the fewest genes encoding these features and is predicted to have evolved from *B. animalis* subsp. *animalis* from extended exposure to a dairy fermentation environment. This readaptation of the *B. animalis* subsp. *lactis* genome may explain the poor colonization abilities that were observed in clinical feeding studies.

The bifidobacterial genome sequences have provided new insights into the biology of these bacteria in the human GI tract. Specifically, they have revealed genomes with extensive features that can be predicted to be important for survival and competition in the large intestine, such as complex carbohydrate utilization and competitive features to protect bifidobacteria from bacteriophages and other bacteria. This is essential to understanding their full role in the gut and how they interact with other members of the microbiota and with the host. Functional investigations of these insights will provide the information needed to effectively utilize this group of bacteria for optimum GI and overall health.

The lack of knowledge regarding the molecular mechanisms of proposed probiotic benefits for bifidobacteria greatly weak-

ens the scientific credibility of health claims. The current explosion in the availability of genome sequences and molecular tools for bifidobacteria provides a means for filling this knowledge void. The immediate future should provide a wealth of information on this important genus and which features are important for both probiotic uses and efficacy. This should enable the identification of strains or groups of strains that can be demonstrated experimentally to carry certain molecular mechanisms relevant to a health claim and also shown clinically to provide health benefits in scientifically valid studies.

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